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Nutrition and feeding in striped bass Morone saxatilis larvae: Lipid and fatty acid requirements and microencapsulated diets

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NUTRITION AND FEEDING IN STRIPED BASS *MORONE SAXATILIS* LARVAE: LIPID AND FATTY ACID REQUIREMENTS AND MICROENCAPSULATED DIETS

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DEDICATION

This dissertation is dedicated to my family for their support and love.

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ABSTRACT

There has been a growing interest in the commercial aquaculture of striped bass, *Morone saxatilis*. Little is known, however, about the nutritional requirements and feeding physiology of striped bass larvae. The objectives of this study were a) to evaluate the efficiency of the live food enrichment techniques in studying the nutritional requirements for polyunsaturated fatty acids (PUFA), phospholipids and free amino acids, b) to determine the ontogenetic changes of lipolytic enzymes, and c) to develop a microencapsulated diet for striped bass larvae as full or partial replacement of live food.

Three enrichment techniques, namely, microencapsulated fish oil, yeast/fish oil emulsion and unicellular algae, Chlorella sp. were evaluated for their efficiency in improving the n-3 PUFA content of *Artemia* nauplii to striped bass larvae. The enrichment of the Artemia nauplii appeared to increase the eicosapentaenoic acid content and enhance the growth of the striped bass larvae.

The uptake and metabolism of ^{14}C-glycine or ^{14}C-phosphatidylcholine labeled liposomes by freshly hatched *Artemia* nauplii were investigated as a new technique for amino acid and phospholipid enrichment. Nearly 80% of the total incorporated ^{14}C-glycine was recovered in the protein fraction of the nauplii, while 85% of the total incorporated ^{14}C radioactivity was found in the lipid fraction. The results of this study suggest that liposomes may be used to enrich Artemia nauplii with phospholipids and free amino acids.

Ontogenetic changes of triacylglycerol hydrolase (TAGH), wax ester hydrolase (WEH) and phospholipase A_2 (PLA_2) were determined in the fertilized eggs and premetamorphosed larvae of striped bass and the larval food *Artemia* using radioassays. PLA_2 was the major enzyme in the eggs followed by TAGH and WEH. The activities of PLA_2, TAGH and WEH in *Artemia* were in the order of PLA_2>TAGH>WEH. The contribution of dietary enzymes to the total lipolytic process was estimated to be less than 6.5% of the total enzyme activity throughout the development. It was estimated that first feeding striped bass larvae had the capacity to digest 47% of their daily lipid ingestion.

The nutritional requirements of marine fish larvae are not well understood primarily due to difficulties in the delivery of biochemically defined diets to the larvae as the only food item. A complex protein-walled microcapsule (CWC) was prepared by incorporating lipid-wall capsules (LWC)containing highly water soluble nutrients along with

other dietary materials in a cross-linked protein-wall microcapsule. The release of amino acid lysine from CWC was determined to be significantly lower than that measured with conventional protein-walled capsules ($P<0.05$). In vitro experiments indicated that the CWC was digested by the crude enzyme extract from striped bass larvae or purified porcine pepsin and trypsin. Results suggest that CWC may be used as a vehicle of nutrient delivery to study the nutritional requirements of marine fish larvae.

Diet acceptability, growth and survival of striped bass larvae fed complex protein-walled microcapsules were investigated in two separate experiments. In both experiment, 7-days post-hatching larvae were fed complex hemoglobin-wall (HWC) or casein-wall (CWC) microencapsulated diets in full or partial replacement of live food Artemia nauplii for two weeks. In both experiments, the acceptability of microencapsulated diets was high. Neither microencapsulated diet support growth when solely fed to the larvae. The results of this study suggested that CWC can be used for partial replacement (60%) of live food without any significant effect on growth and survival of striped bass larvae.

NUTRITION AND FEEDING IN STRIPED BASS (*MORONE SAXATILIS*) LARVAE: LIPID AND FATTY ACID REQUIREMENTS AND MICROENCAPSULATED DIETS

CHAPTER 1

GENERAL INTRODUCTION

The development of an artificial diet that can replace conventional live food organisms has been the primary goal of many aquaculturists in the field of larval nutrition. The formulation and preparation of artificial larval diets require, if not complete, an adequate understanding of the biochemistry and physiology of feeding in fish larvae. Most of our knowledge of the nutritional requirements of fish has been extracted from experiments conducted on juvenile or adult species. Very little is known, however, of the nutritional requirements and feeding physiology of larval fishes.

There has been a growing interest in last two decades from the private, public and academic sectors to commercialize striped bass aquaculture in the United States. In conventional striped bass culture, broodstock spawn in hatcheries and larvae are stocked in fertilized earthen ponds after hatching. In this extensive rearing method, survival rates rarely exceed 10 % by the end of 30 days of feeding (Snow et al, 1980; Houde and Lubbers, 1986). The recent developments in the hormone induced off-season spawning of striped bass have led to the production of larvae that have to be raised in indoor tanks. However, intensive rearing techniques require the use of live food, the dietary value of which is often unpredictable. An artificial diet that is readily acceptable by striped bass larvae is crucial for

reducing the cost of production and increasing dependability. Furthermore, the nutritional requirements of striped bass larvae are not well defined. This dissertation investigates the lipid and fatty acid requirements of striped bass larvae, and the development of an artificial diet.

Lipid and fatty acid nutrition in striped bass larvae

Polyunsaturated fatty acids (PUFA) dominate lipids in marine environment. Marine fish larvae obtain significant amounts of PUFA in their diet from lipid-rich zooplankton. The origin of the PUFA in the marine food web is mainly unicellular algae. Unlike the freshwater fish *Cyprinus carpio* (Watanabe et al. 1975), and *Tilapia nilotica* (Olsen et al. 1990), most marine fish have a limited ability to elongate and desaturate shorter chain fatty acids, such as linolenic acid (18:3n-3) to n-3 PUFA. Therefore, they primarily depend on dietary intake to meet nutritional PUFA requirements. The long-chain fatty acids, eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), play important roles as membrane components and precursors for eicosanoid synthesis. Unlike natural zooplankton, cultured rotifers and *Artemia* are often deficient in PUFA depending on the source and culture conditions. Due to high growth rate and rapid development, marine fish larvae require high concentrations of n-3 PUFA in their diet.

The fatty acid composition of *Artemia* varies considerably with strain and sources (Leger et al., 1986 Watanabe et al. 1980; Watanabe et al., 1978; Yone and Fujii, 1975; Webster and Lovell, 1990). Several studies reported that some geographical strains of *Artemia* lack the essential fatty acids, eicosapentaenoic and docosahexanoic acids (Leger et al., 1986). Newly hatched *Artemia* nauplii are deficient in 22:6n-3, regardless of origin, while the presence of 20:5n-3 heavily depends on the geographical location (Fujita et al., 1979 and Schauer et al., 1980). Therefore, eicosapentaenoic acid (EPA) is the major factor determining the nutritional quality of *Artemia* for marine fish larvae (Watanabe, 1983). A high quality batch of *Artemia* is one in which the EPA content is higher than 4 % of the total fatty acids (Leger et al., 1986).

Various techniques have been developed to improve the fatty acid composition of poor *Artemia* strains by feeding the freshly hatched nauplii diets containing high amounts of PUFA (Watanabe et al. 1978; Watanabe et al. 1980,; Fujita et al. 1980; Watanabe et al. 1982; Sakamoto et al. 1982; Leger et al. 1987; Walford and Lam, 1987; Millamena et al. 1988). Watanabe et al. (1980) reported that the dietary value of *Artemia* nauplii to red sea bream was efficiently improved by feeding the nauplii marine *Chlorella* and ω-yeast which contained high levels of PUFA. In a separate study, larvae of flounder and

red sea bream showed better survival and enhanced growth rates when fed *Artemia* enriched with ω-yeast (Baker's yeast and marine oil emulsion; Watanabe et al., 1982). Sakamoto et al. (1982) reported a measurable increase in 20:5n-3 and 22:6n-3 in the polar lipids of *Artemia*, when fed microencapsulated diets containing cod liver oil and *Tapes* oil, both of which contained high amounts of these fatty acids.

In hatchery trials, the natural food web (or chain) is often simplistically simulated in a small, but highly productive manner. However, under artificial culture conditions the production of unicellular algae is difficult and costly. Therefore, the efficiency of alternative enrichment techniques needs to be determined.

Ontogeny of lipolytic enzymes in striped bass larvae

It is not known whether reduced growth rates obtained using artificial diets are caused by the poor formulation of the microdiets or by their low digestibility, or both. Larval diets that were formulated based on the nutritional requirements of juveniles have consistently failed to support growth and survival of fish larvae. Dabrowski (1979) suggested that initial digestion in larvae was triggered by the action of the enzymes present in the ingested live food organisms. The few attempts that have been made to determine the effects of the inclusion of dietary enzymes into larval

diets have resulted in controversial conclusions (Dabrowski and Glogowski, 1977b; Maugle et al. 1983). Dabrowski and Glogowski (1977b) found no effect of the addition of bovine trypsin to the diet at levels equal to or three times the amount found in natural foods on the survival of common carp fry. However, higher feed conversion efficiency and net protein utilization were obtained from the diets supplemented with bovine trypsin. In a separate study, dietary supplements of α-amylase and bovine trypsin at levels comparable to those of live short-necked clam enhanced the growth of shrimp, *Penaeus japonicus* (Maugle et al. 1983).

Thus far, most enzyme studies have mainly focused on the proteolytic and carbohydrolytic enzymes, such as trypsin, pepsin and amylase (Baragi and Lovell, 1986; Kawaii and Ikeda, 1973; Lauff and Hofer, 1984). Very little information, however, exists on the lipolytic enzymes in fish larvae and their diet. The lipolytic enzymes are particularly important in striped bass larvae, since they obtain significant amounts of energy from endogenous and exogenous lipids. Striped bass larvae are characterized by a single oil globule that supplies >70 % of total egg energy during ontogenetic development (Eldridge et al., 1981). The degradation of the oil globule during development requires significant amounts of lipolytic activity. An unfed striped bass larva retains its oil globule for prolonged periods of time, while a feeding larva utilizes

its oil globule within the first week of feeding (Eldridge et al., 1983). This suggests that unfed striped bass larvae may lack necessary lipolytic enzymes to hydrolyze its maternal lipid reserves, while feeding larvae receive lipolytic enzymes from ingested live food. Therefore, the availability of energy stored in lipids is likely to be dependent on the exogenous supply of lipolytic enzymes from ingested live food, such as *Artemia*. In this respect, the determination of lipolytic activities during the development of striped bass larvae will help researchers understand the physiological processes involved in the digestion of lipids and also provide a basis for formulating the microencapsulated diets.

Microencapsulated diets

Although live food organisms used in larval rearing are superior to any artificial diet developed to date, they have certain disadvantages when used extensively. Firstly, the nutritional quality of live food organisms varies enormously from batch to batch, as determined by changes in their biochemical composition. Secondly, production of live food organisms is costly and time consuming, which is a primary concern in commercial aquaculture. Thirdly, to a certain extent, the size and chemical composition of live food is difficult to fulfill the specific requirements of the larvae of interest. Lastly, live food often contains pollutants (PCBs and heavy metals etc.) and pathogenic organisms (Leger

et al., 1985; Leger et al., 1986)

Thus far, no satisfactory artificial diet has been developed for marine fish larvae which is comparable to live food in their growth effects. However, limited success has been reported with microparticulate and microencapsulated test diets (Kanazawa et al. 1982; Applebaum 1985; Leibowitz et al. 1987; Kanazawa et al.: 1989). Kanazawa et al. (1989) reported that 10-day old red sea bream grew and survived reasonably well when fed micro-bound diets containing a mixture of chicken egg, egg yolk powder, squid meal, clam extract, krill and casein as major protein sources.

A particular problem associated with microparticulate diets besides the low acceptability by fish larvae, is nutrient lost to leaching (Goldblatt et al., 1979). This may be especially severe in larval fishes which do not consume their food for prolonged periods. As a result, water soluble vitamins, free amino acids and certain minerals are rapidly lost through leaching from uncoated, conventionally bound feeds (Goldblatt et al., 1979, Lopez-Alvarado et al. 1994). However, Langdon (1989) successfully reduced the leaching of ^{14}C labelled protein to less than 5 % by the use of modified, cross-linked protein wall capsules. In addition to the reduction in leakage of nutrients from conventional feeds, particle breakdown and clumping were also minimized.

Consequently, higher water quality is obtained with microcapsules, due to the decline of bacteria in the culture medium. Jones et al. (1993) outlined the principal criteria for the microparticulate diets for marine larvae:

1. Particles should be stable in the culture water, retaining their nutritional content until ingested;

2. Diet has to be readily acceptable and palatable by the larvae;

3. Coating material and the content of the diet have to be digestible with no or minimum toxicity;

4. Nutritional content of the diet has to be complete to support good growth and survival of the larvae;

5. Diet should have extended shelf life;

In this respect, microencapsulation technology offers a potential solution to the problems encountered in larval feeding, especially by the use of combined techniques such as, lipid-wall and cross-linked protein wall capsules.

The dietary requirements of fish larvae are usually assumed to be similar to those of adults. The major

difficulty in determining the nutritional requirements of larval fish is the delivery of chemically defined nutrients (or radiolabelled precursors) to the digestive system of the larvae (Langdon and Siegfried, 1984). This is mainly because of the problems associated with nutrient leaching from loosely bound microparticulate diets and the low acceptability of artificial diets by marine fish larvae. However, in the case of larval fish microencapsulation emerges as a promising technique to study the dietary requirements.

The development of a microencapsulated diet for striped bass larvae was first suggested by Kraeuter and Woods (1987). Subsequent attempts made to grow striped bass larvae to the fingerling stage by using artificial diets failed due to the low acceptability of the diets by the larvae (Gallagher, 1987; Tuncer et al., 1990, Webster and Lovell 1990). The low ingestion rate of artificial diets appears to be a major concern in larval feeding. If striped bass aquaculture is to be commercialized, it is critical that an artificial diet that will promote growth comparable to that of live diets be developed.

Objectives

In this dissertation, the nutritional requirement of striped bass larvae for lipids and fatty acids, and the development of a microencapsulated diet were investigated with

specific objectives as follows:

1. Evaluate the efficiency of various PUFA enrichment techniques for *Artemia* and assess their dietary value to striped bass larvae;

2. Determine the feasibility of using liposomes to enrich *Artemia* with phospholipids and free amino acids;

3. Investigate the ontogenetic changes of three major lipolytic enzymes, namely, triacylglycerol hydrolase, wax ester hydrolase and phospholipase A_2 in the fertilized eggs and premetamorphosed larvae of striped bass and live food *Artemia*;

4. Develop a complex microencapsulated diet and evaluate the kinetics of nutrient release, size distribution and *in vitro* digestibility by striped bass larvae;

5. Determine the diet acceptability, growth and survival in striped bass larvae fed complex microencapsulated diets as partial and full replacement of the live food, *Artemia*.

CHAPTER 2

EVALUATION OF OMEGA-3 FATTY ACID ENRICHMENT OF *ARTEMIA* NAUPLII AS FOOD FOR STRIPED BASS *MORONE SAXATILIS* WALBAUM LARVAE.

Introduction

Marine fish larvae require polyunsaturated fatty acids (PUFA) of omega-3 (n-3) family in their diets for growth and development (Fujita et al. 1980; Watanabe et al. 1983). Unlike the freshwater fish *Cyprinus carpio* (Watanabe et al. 1975) and *Tilapia nilotica* (Olsen et al. 1990), most marine fish larvae have limited elongation and desaturation capabilities of shorter chain fatty acids such as linolenic acid (18:3n-3) to n-3 PUFA (Owen et al. 1975; Kanazawa et al. 1979). Therefore, they primarily rely on dietary sources for w3 PUFA, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The dietary requirement for n-3 PUFA is critical in early developmental stages due to the high demand for membrane synthesis where the n-3 PUFA are incorporated (Henderson and Sargent 1985). Studies show that diets deficient in n-3 PUFA resulted in high mortalities and poor growth of the larvae of red sea bream (*Pagrus major*) (Watanabe et al. 1980), striped bass (Webster and Lovell 1990a) and hybrid striped bass (*Morone saxatilis x Morone crysops*) (Tuncer and Harrell 1992).

Although striped bass are tolerant to a wide range of salinities, they have demonstrated a fatty acid requirement similar to that of a marine species (Webster and Lovell 1990a, Tuncer and Harrell 1992). EPA was found to be essential for

striped bass larvae at levels higher than 5 % of total fatty acids (Webster and Lovell 1990a). Lower levels of EPA resulted in poor growth and survival and the appearance of deficiency symptoms such as dysfunctional swim bladder in the larvae. In a separate study, recovery of striped bass hybrid larvae from shock syndrome was found to be positively correlated to the amount of n-3 PUFA in the diet (Tuncer et al. 1992).

Nutritional quality of *Artemia* for marine fish larvae is determined by the fatty acid composition of its lipid classes (Watanabe et al. 1980; Leger et al. 1986). It has been reported that the fatty acid content of *Artemia* varies enormously among different strains and sources (Watanabe et al. 1982; Fujita et al. 1980; Leger et al. 1986; Webster and Lovell 1990b). EPA appears to be the critical factor determining the nutritional quality of *Artemia* for marine fish larvae. Regardless of origin, DHA is nearly absent in *Artemia* while EPA shows remarkable variations with geographical strains. Newly hatched *Artemia* nauplii are conventionally used as a first food for striped bass larvae. Webster and Lovell (1990b) have evaluated the dietary value of *Artemia* nauplii from different sources for striped bass larvae and found great variation in growth and survival rates.

The fatty acid composition of *Artemia* nauplii can be

modified by feeding diets rich in n-3 PUFA. Various enrichment techniques have been developed to increase the n-3 PUFA content of *Artemia* nauplii to levels that would satisfy the essential fatty acid requirement of marine fish larvae. Currently used enrichment techniques include 1) emulsified diets containing marine oils rich in n-3 PUFA (Watanabe et al. 1980, 1982; Leger et al. 1987), 2) unicellular algae (Watanabe et al. 1980; Millamena et al. 1988), and 3) microencapsulated diets containing high concentrations of w3 PUFA (Sakamoto et al. 1982; Walford and Lam 1987). Lemm and Lemarie (1991) and Tuncer and Harrell (1992) successfully enriched *Artemia* nauplii with n-3 PUFA using a self-emulsifying n-3 PUFA concentrate and enhanced its nutritional quality for striped bass larvae. However, efficiency of various enrichment techniques for marine fish larvae has not been compared in a single study. In this study, we compared the efficiency of three enrichment techniques; namely, gelatin-acacia microcapsules containing menhaden oil, unicellular algae *Chlorella sp.* (marine type) and a yeast-menhaden oil emulsion to enhance the nutritional quality of *Artemia* from Great Salt Lake which contained low levels of EPA for striped bass larvae.

Material and Methods

Feeding conditions

I. *Enrichment* - Two strains of *Artemia* cysts (Great Salt

Lake and San Francisco Bay origin) were purchased from San Francisco Bay Brand Inc. (Newark, CA USA). The cysts were chemically decapsulated to eliminate contamination of the rearing media with hatching debris (Sorgeloos et al. 1977) and were hatched in 20 l glass bottles (24 ppt, 26°C, 48 hours). Hatched nauplii were rinsed and divided into 4 1 beakers in duplicates containing 1 µm filtered estuarine water (24 ppt) for enrichment. The enrichment period was 12-24 hours. The three enrichment diets were prepared by feeding *Artemia* nauplii from Great Salt Lake Origin with; (**CHL**) marine type *Chlorella sp.* at a density of $15x10^6$ cells/ml, (**YMO**) yeast and menhaden oil emulsion containing 0.1 g Baker's yeast and 0.1 ml menhaden oil emulsified in 50 ml distilled water, and (**GAC**) gelatin-acacia microcapsules (5-20 µm) containing menhaden oil at a density of $10x10^3$ capsules/ml.

II. *Feeding* - Five days post-hatched striped bass larvae were obtained from Brookneal State Hatchery, Virginia. Larvae were kept in the laboratory in fresh water at 21 °C overnight (ca. 16 hr). On day six, 1400 larvae were transferred to each of 12 individual 70 liter rearing tanks to provide a density of 20 larvae/l. A 600 µm mesh screen was placed at the bottom of each conical tank to allow uneaten food particles to leave the culture media. Culture water was recirculated through a 5 µm filter to eliminate the fecal particles and uneaten *Artemia* nauplii. Salinity, temperature and water flow were 2

ppt, 21 ± 1 °C, 0.8 L/min, respectively. Ammonia was removed by a biofilter system which consisted of preconditioned, crushed oyster shells. The culture tanks were continuously aerated. On 7 days after hatching (hereafter referred to as "day 0"), feeding was initiated (5 nauplii/ml) and continued for 21 days. Unenriched *Artemia* nauplii of Great Salt Lake (**GSL**) and San Francisco Bay (**SFB**) origin were used as controls. A starved group was included in the experiment to compare fed and unfed larvae. Thus, there were a total of 6 treatments in duplicates (2 culture tanks) which were randomly assigned to 12 experimental tanks.

Larval fish measurement

Ten larvae were sampled for total length and wet weight, on days 0 and 7 with 25 larvae sampled on days 14 and 21 from each tank. Total length and wet weight were determined on individual larva after blotting dry on a paper towel. Total length was measured using an optical micrometer and then weighed to the nearest tenth of a milligram. At the same time, 10-15 larvae were sampled into a glass test tube for lipid and protein analyses. Survival rates expressed as percentages were determined by counting the number of fish that survived at the end of the experiment, taking into account the samples withdrawn during feeding.

Lipid and fatty acid analyses

Total lipids were extracted from pooled *Artemia* nauplii and larval tissues from each tank with chloroform-methanol-water (2:2:1) according to the method of Bligh and Dyer (1959). Total lipid content of the sample was measured by the method of Holland and Gabbot (1971) and the amount was expressed as a percentage of dry weight.

Total lipids were transesterified with methanol and boron trifluoride (Cosper and Ackman 1983). Separation of the fatty acid methyl esters (FAME) was carried out on a gas liquid chromatograph (GLC Varian 3300, Sugar Land, TX) equipped with a flame ionization detector, using a fused silica capillary column coated with OV-351 (30 m * 0.32 mm i.d., J & W Scientific Inc., Folsom, CA). The column was temperature-programmed from 120 to 180 °C at 12 °C/min and from 180 to 220 °C at 6 °C/min; injector and detector temperatures were 220 and 240 °C, respectively; the flow rates of compressed air and hydrogen were 300 ml/min and 30 ml/min, respectively; helium was used as the carrier gas (1.5 ml/min).

Identification and quantitation of FAMEs was based on the comparison of the sample retention time to those of known standards (18:0, 21:0, 20:5n-3, 22:6n-3). Purified menhaden oil was also used as a secondary standard for identification. FAME peaks were quantified by computer analysis

(Chromatochart-PC, Interactive Microware, Inc., State College, PA) connected to the GLC. The results are corrected with the response factors of purified standards and expressed as a percentage of the total fatty acid methyl esters.

Protein analysis

The method of Lowry et al. (1951) was used for the protein analysis of the larvae. Freeze dried samples were hydrolyzed in 1 N NaOH at 110 °C for 4 hours. Bovine serum albumin was used as a standard.

Preparation of gelatin-acacia capsules

Gelatin-acacia microcapsules were prepared by using methods of Green and Schleicher (1957).

Statistical analysis

Wet weight and total length data were found to have non-homogenous variance and were $\log_{10}$ transformed for ANOVA. ANOVA blocked by tank for both wet weight and total length data showed that there were no significant tank effects (P=0.81, P=0.26 respectively). Therefore, the data were pooled and one-way ANOVA was employed for statistical comparisons. Survival, protein, lipid and fatty acid data were arc sin transformed, then the differences between treatments were compared using one-way ANOVA. Pairwise comparisons were made with Scheffe's test using SPSS-X

statistical package. Differences were deemed statistically significant when $P \leq 0.05$.

Results

Growth and survival of larvae

Larvae fed unenriched GSL nauplii were significantly lighter and smaller than those of the enriched groups YMO, GAC, CHL and unenriched SFB nauplii at the end of the experiment (Table 1). There were no significant differences in wet weight and total length among larvae fed enriched GSL nauplii (YMO, GAC, CHL) and unenriched SFB nauplii. On day 7, larvae fed unenriched GSL nauplii showed significantly slower growth than the other groups. This trend continued throughout the experiment. Similar results were observed with total length data (Table 1).

Unfed larvae were all dead by day 8. Survival rates for experimental groups varied between 21.3 % (GAC) and 30.3 % (YMO) and did not follow the trend that can be observed with wet weight and total length (Table 1). There were no significant differences in survival among all groups at the end of the experiment.

Lipids and fatty acids of *Artemia*

All three enrichment techniques effectively improved the fatty acid composition of *Artemia* nauplii from Great Salt Lake

(GSL) compared to unenriched nauplii of the same origin. Linolenic acid (18:3n-3) and EPA (20:5n-3) increased significantly in the groups enriched with *Chlorella sp.* (CHL), yeast and menhaden oil emulsion (YMO) and gelatin-acacia microcapsules (GAC) (Table 2). YMO and GAC enriched nauplii had significantly higher EPA levels than CHL enriched nauplii which appeared to be less efficient than GAC and YMO enrichments. Although SFB nauplii did not receive any enrichment, EPA levels were comparable (5.1 % of FAME) to those of YMO and GAC and higher than CHL nauplii. Oleic acid (18:1n-7 and 18:1n-9) was the most representative fatty acid while DHA was not detectable in all groups.

Protein, lipid and fatty acid composition of striped bass larvae

Percent protein content (% dry weight-DW) of the larvae on day 0 was 55.8 % and dropped drastically on day 7 in unfed larvae (13.2 %). There were no significant differences in protein content among all the groups at the termination of the experiment (Table 1).

Initial lipid content (% DW) of the larvae was 40.9 % and dropped remarkably at the end of the experiment (5-7.5 % by day 21) in all groups except the larvae fed on unenriched GSL nauplii which seemed to preserve its lipid reserves for extended periods - 23.1 % on day 14 (Table 1). Unlike fed

larvae, unfed larvae had the highest lipid content on day 7 due to the preservation of oil globule. The oil globules of unfed larvae were intact and formed a greater portion of body mass on day 14 when stained with Oil Red O (results not shown).

The fatty acid composition of striped bass larvae at the end of the experiment reflected the fatty acid composition of the diet *Artemia* nauplii, with the exception of the larvae fed unenriched GSL nauplii (Table 3). Day 0 larvae contained relatively higher levels of DHA (12.7 %) which decreased sharply after feeding. EPA levels were significantly higher in all feeding larvae than their diets, being highest in larvae that fed YMO nauplii. Although the GSL nauplii did not contain higher amounts of EPA, larvae fed GSL nauplii had significant amounts of EPA (9.0 %). Linolenic acid also increased from 5.0 % in initial larvae to 11-13 % in feeding larvae.

Table 1. Wet weight (mg), total length (mm), total lipids (% of dry weight), total protein (% of dry weight) and survival (%) of striped bass larvae fed enriched and unenriched *Artemia* nauplii for 21 days. UNFED=starved, GSL=unenriched Great Salt Lake nauplii, SFB=unenriched San Francisco Bay nauplii, YMO=yeast and menhaden oil enriched, GAC=gelatin-acacia capsules enriched, CHL=*Chlorella sp.* enriched Great Salt Lake nauplii. Values are the means ± standard error. Means with identical superscripts are not significantly different among values in the same row.

	Day	UNFED	GSL	CHL	YMO	GAC	SFB	N
Wet Weight (mg)	0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	n=10
	7	0.5^{c} ± 0.0	2.9^{a} ± 0.2	3.7^{b} ± 0.1	3.5^{ba} ± 0.2	4.0^{b} ± 0.2	3.4^{ba} ± 0.2	n=20
	14	-	7.3^{a} ± 0.4	8.8^{ab} ± 0.4	9.7^{b} ± 0.4	10.7^{b} ± 0.6	9.4^{ab} ± 0.6	n=50
	21	-	19.0^{a} ± 1.1	29.4^{b} ± 1.2	29.2^{b} ± 2.0	32.5^{b} ± 1.6	30.0^{b} ± 2.1	n=50
Total Length (mm)	0	5.4 ± 0.3	5.4 ± 0.3	5.4 ± 0.3	5.4 ± 0.3	5.4 ± 0.3	5.4 ± 0.3	n=10
	7	5.4^{a} ± 0.1	7.7^{b} ± 0.2	7.6^{b} ± 0.2	7.9^{bc} ± 0.2	8.2^{bc} ± 0.3	8.0^{bc} ± 0.2	n=20
	14	-	10.2^{a} ± 0.4	11.7^{b} ± 0.3	11.0^{b} ± 0.3	11.3^{b} ± 0.3	11.1^{b} ± 0.3	n=50
	21	-	14.3^{a} ± 0.5	15.9^{b} ± 0.4	15.2^{b} ± 0.9	16.2^{b} ± 0.4	15.4^{b} ± 0.6	n=50
Total lipids (%)	0	40.9	40.9	40.9	40.9	40.9	40.9	n=1
	7	79.6^{a} ± 2.1	22.2^{b} ± 0.7	17.6^{b} ± 3.4	21.1^{b} ± 7.8	22.2^{b} ± 0.0	20.8^{b} ± 3.0	n=2
	14	-	23.1^{a} ± 8.0	15.4^{a} ± 5.7	13.1^{a} ± 2.7	14.5^{a} ± 0.1	13.8^{a} ± 0.9	n=2
	21	-	6.1^{a} ± 0.7	5.0^{a} ± 0.4	7.2^{a} ± 0.2	7.5^{a} ± 1.0	5.8^{a} ± 0.6	n=2
Total Protein (%)	0	55.8	55.8	55.8	55.8	55.8	55.8	n=1
	7	13.2^{a} ± 0.5	39.2^{b} ± 1.0	22.4^{a} ± 0.0	27.9^{ab} ± 1.7	18.3^{a} ± 5.3	40.7^{b} ± 2.5	n=2
	14	-	68.2^{ab} ± 6.2	57.0^{b} ± 1.9	50.5^{b} ± 9.1	38.9^{a} ± 3.2	64.4^{b} ± 6.3	n=2
	21	-	38.7^{a} ± 15.9	54.9^{a} ± 1.5	45.7^{a} ± 5.8	47.0^{a} ± 6.4	49.5^{a} ± 1.8	n=2
Survival (%)	21	-	29.8^{a} ± 5.6	25.9^{a} ± 1.6	30.3^{a} ± 10.8	21.3^{a} ± 4.7	24.8^{a} ± 3.5	n=2

Table 2. Fatty acid composition (percent of total FAME) of enriched and unenriched *Artemia* nauplii. Values are the means of two replicates. Abbreviations are as in table 1.

Fatty Acids	GSL	CHL	YMO	GAC	SFB
14:0	1.2	0.6	0.6	0.5	0.4
14:1	1.0	tr	0.1	tr	0.3
15:0	0.6	tr	0.2	0.1	0.2
16:0	19.2	13.0	11.2	10.4	10.2
16:1w7[x]	6.2	2.9	3.8	2.9	4.3
16:2w6[x]	1.1	1.4	1.3	1.4	1.3
16:3w6	1.8	0.7	0.2	0.6	0.9
16:4w3	1.1	0.4	0.5	0.6	0.5
17:0	1.9	0.9	0.8	0.8	0.6
18:0	11.2	8.6	9.5	8.6	8.5
18:1w9	19.6	20.6	20.7	21.1	22.4
18:1w7	11.9	16.6	15.7	17.3	17.1
18:2w6	3.6	5.9	4.1	4.2	3.5
18:3w3	11.4[b]	18.3[a]	18.8[a]	18.9[a]	14.0[ab]
18:4w3	1.4	3.1	2.3	3.2	2.8
20:1	0.4	tr	0.3	0.2	0.3
20:4w6	0.7	0.9	1.0	1.2	2.3
20:5w3	1.7[a]	3.7[b]	6.8[c]	5.4[c]	5.1[c]
Unidentified	4.0	2.4	2.1	2.6	5.3
Total w3	15.6[a]	25.5[b]	28.4[b]	28.1[b]	22.4[b]
Total w6	7.2	8.9	6.6	7.4	8.0
w3/w6	2.2	2.9	4.3	3.8	2.8

[x] Contains small amounts of other isomers

tr Trace (<0.1%)

[a,b,c] Means with different superscripts are significantly different ($P<0.05$).

Table 3. Fatty acid composition (percent of total FAME) of striped bass larvae fed enriched and unenriched *Artemia* nauplii on day 0 and day 21. Values are the means of duplicates. Abbreviations are as in Table 1.

Fatty Acid	GSL	CHL	YMO	GAC	SFB
14:0	0.4	0.5	0.5	0.4	0.6
14:1	0.2	0.2	0.2	0.3	0.2
15:0	0.2	0.2	0.2	0.3	0.2
16:0	14.8	13.6	14.5	13.9	15.6
16:1w7[x]	3.2	3.3	3.5	3.4	4.2
16:2w6[x]	0.9	0.9	0.6	1.2	0.9
16:3w6	1.4	1.4	1.0	1.3	1.3
16:4w3	0.0	0.1	0.7	0.1	0.0
17:0	1.0	1.0	0.9	1.1	0.9
18:0	9.3	9.0	9.7	9.3	9.0
18:1w9	19.3	19.7	17.6	19.4	20.7
18:1w7	10.3	9.5	9.2	9.2	10.6
18:2w6	5.7	5.3	4.4	5.5	5.1
18:3w3	13.7[a]	13.8[a]	11.0[a]	14.5[a]	13.2[a]
18:4w3	1.4	1.4	1.1	1.5	1.5
20:0	0.2	0.3	0.1	0.3	0.2
20:1w9	0.7	0.9	0.6	0.7	0.7
20:2w6	0.1	0.3	0.2	0.3	0.0
20:3w3	0.2	0.4	0.2	0.4	0.2
20:4w6	5.1	4.5	4.3	4.4	4.7
20:5w3	9.0[b]	8.2[b]	11.7[c]	8.5[b]	7.6[b]
22:1	0.0	0.2	0.2	0.0	0.0
22:5w3	0.1	0.6	1.2	tr	tr
22:6w3	0.8[b]	1.3[b]	3.3[b]	1.0[b]	0.9[b]
Unidentified	2.0	3.4	3.1	3.0	1.7
Total w3	25.2[a]	25.8[a]	29.2[a]	26.0[a]	23.4[a]
Total w6	13.2	12.4	10.5	12.7	12.0
w3/w6	1.9	2.1	2.8	2.0	2.0

[x] Contains small amounts of other isomers.

tr Trace (<0.1%).

[a,b,c] Different superscripts are significantly different ($P<0.05$).

Discussion

All three techniques appeared to be effective to enrich *Artemia* nauplii with n-3 PUFA to levels comparable with previous studies (Watanabe et al. 1980, 1982; Walford and Lam 1987; Sakamoto et al. 1982; Millamena et al. 1988) and satisfied the nutritional requirement of striped bass larvae for n-3 PUFA. Higher EPA content of YMO fed nauplii could be attributed to the high ingestibility of the smaller sized particles of yeast. Watanabe et al. (1980) used Baker's yeast-cuttlefish liver oil emulsion to increase the EPA and DHA content of newly hatched *Artemia* nauplii from 1.6 % to 4.5 % of total FAME after 24 hours of enrichment. In a separate study, Leger et al. (1987) enriched *Artemia* nauplii with n-3 PUFA within 24 hrs using an emulsified diet. Although the efficacy of emulsified diets is higher than the other enrichment techniques, they are often associated with bacterial contamination of the enrichment media, thus resulting in low oxygen concentrations and high mortalities of *Artemia* during enrichment (Leger et al., 1987).

Protein-wall microcapsules have been successfully used to enrich *Artemia* nauplii with n-3 PUFA (Walford and Lam, 1987), cholesterol, amino acids, and nucleic acids (Sakamoto et al., 1982). In this study, EPA content of *Artemia* nauplii enriched with gelatin-acacia microcapsules (5-20 μm)

containing menhaden oil was not significantly different from that of yeast-menhaden oil emulsion enriched nauplii. Walford and Lam (1987) increased the EPA content of *Artemia* nauplii from 11.2 % to 12.2 % of FAME within 8 hours of feeding a commercial microencapsulated diet after feeding Baker's yeast for 20 hrs. Sakamoto et al. (1982) fed *Artemia* nauplii protein-wall microcapsules (5-10 μm) containing cod liver oil. In their study, it was indicated that EPA was present at 1 % of total FAME in newly hatched nauplii and increased to 9.2 % in the polar lipid fraction after 18 days.

EPA content of *Artemia* nauplii appeared to be the critical factor in determining the growth of striped bass larvae. A number of studies have indicated that striped bass larvae require EPA at concentrations higher than 5 % of total FAME in their diet (Webster and Lovell 1990a; Lemm and Lemarie 1991; Tuncer and Harrell, 1992). Webster and Lovell (1990a) reported lower growth and survival of striped bass larvae when fed *Artemia* nauplii with EPA content lower than 3 % of total FAME. However, in the present study survival rates were not significantly different among treatments which contained high or low concentrations of EPA; 33 % of the larvae survived when fed *Artemia* nauplii from Great Salt Lake that had 1.7 % EPA concentration. Lemm and Lemarie (1991) reported 23 % survival of striped bass larvae when

fed nauplii containing 2.6 % EPA and 5 % survival when fed nauplii containing 3.8 % EPA of total FAME. It appears that survival is not as indicative of essential fatty acid deficiency as growth unless the larvae are exposed to shock syndrome (Tuncer and Harrell, 1992).

A number of studies have indicated that striped bass may not be capable of elongating and desaturating (or bioconversion) shorter chain precursors to n-3 PUFA (Webster and Lovell, 1990a, Lemm and Lemarie, 1991; Tuncer and Harrell, 1992). Lemm and Lemarie (1991) and Tuncer and Harrell (1992) suggested a limited bioconversion rate that is unlikely to supply the high PUFA demand for growth of striped bass larvae. In their studies, however, the EPA concentration of striped bass larvae was higher in all treatments than that of diet *Artemia* nauplii, indicating a possible bioconversion of linolenic acid to EPA. Moreover, the concentration of linolenic acid in larvae was proportionally lower than those of the diet *Artemia*, perhaps due to the utilization of this fatty acid in n-3 PUFA synthesis. Similar results were also observed in our study in that striped bass larvae had higher EPA concentration than those of their diets. Remarkably, striped bass larvae fed unenriched GSL nauplii containing 1.7 % EPA had 9.0 % EPA content, possibly due to the accumulation of this fatty acid from dietary sources and bioconversion of linolenic

acid to EPA. Although DHA was absent in the diet *Artemia*, there were small amounts of this fatty acid present in the larvae. Elongation and desaturation of linolenic acid to DHA in striped bass larvae was also suggested by Martin et al., (1984). With the exception of linolenic acid, EPA and DHA, the relative concentrations of all other fatty acids in the diet *Artemia* were reflected in the fatty acid composition of the larvae. In fish, desaturation and elongation pathways involve the utilization of linolenic acid in the production of EPA and DHA which are ultimately incorporated in membrane phospholipids and prostaglandins (Henderson and Sargent, 1985). A decrease in the concentration of linolenic acid followed by an increase in EPA and DHA concentration suggests a possible conversion of linolenic acid to n-3 PUFA.

The nutritional quality of *Artemia* is often unpredictable, thus making high quality strains costly. Therefore, it is advisable that the fatty acid composition of *Artemia* be determined prior to feeding the larvae and an appropriate enrichment be employed when necessary. Among all the enrichment techniques investigated in this study, microencapsulated diets emerge as the most promising technique, because of their ease of application, reasonable costs, minimum contamination of the enrichment media by bacteria and their ability to encapsulate a wide variety of

nutrients.

CHAPTER 3

UPTAKE AND METABOLISM OF LIPOSOMES BY *ARTEMIA* NAUPLII

Introduction

Brine shrimp *Artemia* sp. and rotifers *Brachionus plicatilis* are the most commonly used live food organisms for the culture of marine fish larvae. The nutritional quality of live food is determined by their fatty acid composition and varies greatly among sources and strains (Leger et al., 1986). The dietary requirement of marine fish larvae for polyunsaturated fatty acids (PUFA) of the n-3 family is well documented (Fujita et al., 1980; Watanabe et al., 1983). Some strains of *Artemia* are notorious for their low content of essential PUFA, especially eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. A number of methods have been developed to modify the fatty acid composition of live foods with varying degrees of efficiency (Watanabe et al., 1980, Sakamoto et al., 1982; Leger et al., 1987; Ozkizilcik and Chu, 1994a). Several studies have successfully demonstrated the use of unicellular algae (Watanabe et al., 1980), emulsified (Leger et al., 1987) and microencapsulated diets to enrich *Artemia* with n-3 PUFA (Sakamoto et al., 1982; Walford and Lam, 1987; Ozkizilcik and Chu, 1994a). Although most strains of *Artemia* require laborious and costly enrichment to increase the n-3 PUFA content, the beneficial effects of enrichment make this process worthwhile. In addition to the use of

enrichment in fatty acid nutrition, live food mediated-delivery (or bioencapsulation) of therapeutic drugs has emerged as a new tool for disease treatment in larval culture (Verpraet et al., 1992). Live food mediated delivery is particularly important for marine fish larvae that do not accept artificial diets as first food.

Liposomes are spherical vesicles in which an aqueous volume is entrapped by a membrane composed of lipid molecules, usually in the form of phospholipids. Phospholipids form concentric bilayers when dispersed in an aqueous medium enclosing the aqueous material in the core, as well as within the bilayered lamellae. They differ from lipid microspheres and lipid-walled microcapsules as they primarily consist of phospholipids. Liposomes are identical to natural biomembranes (Bangham, 1972) with a size range from a few nanometers to several micrometers. The compatible size range and complete digestibility of liposomes (Lasic, 1992) make them a good vehicle for the study of nutritional requirements of aquatic filter feeders (Parker and Selivonchick, 1986). Parker and Selivonchick (1986) successfully used liposomes as a nutrient delivery system for Pacific oyster (Crassostrea gigas). They demonstrated that liposomes containing radio-labeled phosphatidylcholine, cholesterol, glucose and amino acids were incorporated into the tissues of Pacific oyster

juveniles. Nevertheless, liposomes have yet to find extensive application in the study of nutritional requirements in marine organisms. In this study, we have investigated the feasibility of using liposomes to modify the phospholipid and free amino acid composition of *Artemia* nauplii. Additionally, an attempt was made to determine the uptake, digestion and subsequent metabolism of dietary phospholipids and free amino acids in *Artemia*.

Materials and Methods

Preparation of liposomes

Liposomes were prepared according to the method described by New (1990). Purified phosphatidylcholine (PC), cholesterol, phosphatidylglycerol and menhaden oil (100:40:10:20 mg, respectively) were dissolved in 5 ml of chloroform:methanol (2:1 v/v) in a round-bottomed flask. The solvent was evaporated at 30 °C under nitrogen in a rotary evaporator (Rotavapor-R, Brinkman, NY). Five ml of physiological saline (0.9 % NaCl) solution were introduced into the flask along with 1 g of glass beads. The suspension was rotated for 30 min at room temperature and was kept for 2 h for complete swelling. Liposomes were collected after repeated washings and centrifugations at 14,000 rpm. Mean size of liposomes was 2.6 ± 1.8 (s.e., n=50) µm, with a size range from 0.4 to 10 µm.

Liposomes containing radio-labeled phosphatidylcholine (^{14}C-PC) were prepared in the aforementioned manner by adding 1 µCi of L-α-dipalmitoyl-[2-palmitoyl-1-^{14}C] phosphatidylcholine (55.5 mCi/mmol; NEN Research Products, MA) into the chloroform:methanol solution with bulk non-labeled PC. Free amino acid labeled liposomes were prepared by adding 2.5 µCi [1-^{14}C]-glycine (52.9 mCi/mmol; NEN Research Products, MA) into physiological saline. Glycine labeled liposomes were repeatedly washed and centrifuged until no activity was measured in the supernate. Approximately 20 % of the ^{14}C-glycine was found to be entrapped in the liposomes.

Enrichment

Five grams of *Artemia* cysts of Great Salt Lake origin (San Francisco Bay Brand, CA) were hatched in 1 l Imhoff cones containing filtered (1 µm) estuarine water (salinity=24 ppt) at 28 °C under constant illumination. Nauplii were collected immediately after hatching using 150 µm filters, rinsed well with 1 µm filtered estuarine water and divided into 50 ml plastic centrifuge tubes in triplicates, giving a density of 450 nauplii/ml. Four experimental groups consisted of two labeled and two non-labeled liposomes fed to the nauplii separately under continuous aeration. Labeled liposomes contained ^{14}C-glycine (10^4 dpm/ml of culture medium) or ^{14}C-PC ($2x10^4$

dpm/ml of culture medium). Non-labeled liposomes contained protein (100 mg/ml bovine serum albumin), glucose (50 mg/ml), and vitamin+mineral mixture (2 mg/ml), or physiological saline. Unfed *Artemia* nauplii served as the control. Non-labeled liposomes were added to the enrichment media at a concentration of 1.2 mg total lipid/ml.

Protein, lipid and fatty acid analysis

Artemia nauplii were sampled on 150 µm filters 24 and 48 h after enrichment with liposomes and rinsed well with filtered estuarine water. Total lipids were extracted from *Artemia* nauplii with chloroform:methanol:water (2:2:1 v/v/v) containing 0.01 % (w/v) antioxidant (BHT), according to the method of Bligh and Dyer (1959). The total lipid content of each sample was measured according to the colorimetric method described by Holland and Gabbott (1971) and expressed as µg lipid per mg wet tissue. Solvent insoluble residue was dried under a stream of nitrogen and hydrolyzed with 1 N NaOH. Total protein content was quantified according to the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Lipid classes were separated by high performance thin layer chromatography (HPTLC) on silica gel plates (Whatman HP-K) using a solvent mixture of hexane:diethyl ether:acetic acid (80:20:1 v/v/v). After developing, the plates were

dried briefly and charred with 3 % cupric acetate in 8 % aqueous phosphoric acid at 180 °C for 20 min (Fewster et al., 1969). Lipid fractions were quantified with a GS-300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco). Known quantities of purified standards were run in parallel (N=3) at a linear range of 1-8 µg of each lipid class and the response factor of each standard was calculated. The results were corrected and expressed as µg/mg wet weight.

Total lipids were transesterified with methanol and boron trifluoride (Cosper and Ackman, 1983). Separation of the fatty acid methyl esters (FAME) was carried out on a gas liquid chromatograph (GLC Varian 3300) equipped with a flame ionization detector, using a fused silica capillary column coated with SP-2330 (30 m * 0.2 mm i.d., Supelco Inc., Bellafonte, PA). The column was temperature-programmed from 120 to 180 °C at 12 °C/min and from 180 to 220 °C at 6 °C/min; injector and detector temperatures were 220 and 240 °C, respectively; the flow rates of compressed air and hydrogen were 300 ml/min and 30 ml/min, respectively; helium was used as the carrier gas (1.5 ml/min).

Identification and quantitation of FAME were based on the comparison of the sample retention time to those of authentic standards. Purified menhaden oil was also used as

a secondary standard for identification. FAME peaks were quantified by computer integration (Chromatochart-PC, Interactive Microware, Inc.) connected to the GLC. The results were corrected with the response factors of purified standards and expressed as percentages of the total fatty acid methyl esters.

Analysis of ^{14}C-PC and ^{14}C-glycine uptake by *Artemia* nauplii

Incorporation of ^{14}C-PC and ^{14}C-glycine was determined in chloroform, methanol fractions and NaOH hydrolysate of the nauplii. These measurements represented lipids, water soluble compounds and bound-protein fractions, respectively. The distribution of radioactivity among lipid classes was measured after the separation of total lipids on conventional TLC plates using the technique indicated as above. Lipid classes were visualized under short wavelength UV, after spraying the plates with 0.1 % (w/v) 2,7-dichlorofluoroscein in 95 % (v/v) ethanol. Lipid classes identification was based on the retention times of authentic lipid standards run in parallel and corresponding silica layers were scraped into scintillation vials for counting. Activities were measured in 5 ml of a biodegradable scintillation cocktail suitable for TLC scrapings (Ecolite +, ICN Biomedicals, CA) using a Beckman LS 5000TD liquid scintillation counter.

Uptake of liposomes by *Artemia* nauplii was calculated as:

$$\text{Uptake (\%)} = \frac{\text{Total radioactivity of nauplii(chloroform+methanol+NaOH fractions)}}{\text{Total radioactivity of labeled liposomes added to the culture medium}} \times 100$$

Statistical analysis

Radioactivity counts, total protein, lipid classes and major fatty acid components were included in the statistical analysis. Percentages were arc sin transformed while $\log_{10}$ transformation was employed for radioactivity counts and biochemical composition data. Two-way ANOVA was employed to determine the effects of time (24 and 48 h) and treatment followed by pairwise multiple comparison of means (Tukey) using the Systat^{R} statistical package.

Results

Uptake and distribution of ^{14}C labeled liposomes

The results of this study indicated that the newly hatched *Artemia* nauplii ingested liposomes. Approximately 15% (0.30 nmole) of the ^{14}C-glycine and ^{14}C-PC were incorporated in *Artemia* nauplii within 24 h (Fig. 1). Total incorporated ^{14}C-PC activity significantly decreased ($P<0.05$) from 14% (0.42 nmole) to nearly 11% (0.33 nmole) after 48 h. Total uptake of ^{14}C-glycine, on the other hand, increased slightly (0.33 nmole) after 48 h.

The incorporation of both ^{14}C-glycine and ^{14}C-PC in chloroform, methanol fractions and NaOH hydrolysate varied with time (Fig. 2). A significant proportion (80%) of the incorporated ^{14}C-glycine label was recovered in the NaOH hydrolysate after 24 h and increased slightly ($P>0.05$) after 48 h. Nearly 17% of the ^{14}C-glycine radioactivity was present in MeOH fraction at 24 h and dropped significantly at 48 h. The chloroform fraction contained a small proportion (3%) of the total ^{14}C-glycine label. In contrast, approximately 85% of the total incorporated ^{14}C-PC radioactivity was recovered in the chloroform fraction at 24 h which was followed by a significant ($P<0.05$) decrease at 48 h. The methanol fraction and NaOH hydrolysate contained a small proportion of ^{14}C-PC radioactivity after 24 h. This increased significantly after 48 h.

The distribution of ^{14}C-glycine and ^{14}C-PC activity among lipid classes of nauplii were markedly different (Table 1). At 24 h, all (100 %) of the ^{14}C-glycine label was incorporated into the phospholipids (PL). At 48 h, however, nearly 40% of this activity was associated with triacylglycerols (TAG). Phospholipids and TAG accounted for 97% of the total radioactivity of lipids in the nauplii fed ^{14}C-PC liposomes after 24 h. Phospholipid and TAG counts decreased significantly after 48 h. Percent distribution of TAG and PL, on the other hand, were not significantly

different between 24 and 48 h. Free fatty acids and wax/cholesteryl esters counts increased significantly after 48 h.

Protein, lipid and fatty acid composition of unfed and liposomes (non-labeled) fed *Artemia*

The protein, total lipid and lipid class composition of fed and unfed nauplii after 24 and 48 hours are shown in Table 2. With the exception of cholesterol, all components significantly decreased after time 0. Total lipid content of the liposome fed nauplii was significantly higher than that of the unfed nauplii at 24 h. This was mainly due to the differences in TAG content. Phospholipids, on the other hand, did not show any apparent difference between the fed and unfed nauplii at 48 h, and were slightly lower in the unfed nauplii at 24 h ($P<0.05$). There was a slight affect of using dietary supplements in liposomes on the protein composition of the nauplii. The nauplii fed liposomes supplemented with dietary compounds had higher protein content than nauplii fed liposomes containing only physiological saline ($P<0.05$) after 24 h. Total lipid content of liposome fed nauplii was significantly higher than the unfed nauplii after 24 h. After 48 h, however, there were no significant difference in total lipid and protein content between liposome fed and unfed nauplii ($P>0.05$).

The fatty acid analysis of non-labeled liposomes indicated that palmitic (16:0), oleic (18:1n-9) and linoleic (18:2n-6) acids were the most abundant fatty acids (Table 3). There were only small amounts of arachidonic (2.9%, 20:4n-6) and eicosapentaenoic (%1.8, 20:5n-3) acids present. The fatty acid composition of the total lipid of the nauplii in all treatments was dominated by palmitic, oleic and linolenic (18:3n-3) acids. There were no remarkable changes in the fatty acid components between treatments, with the exception of arachidonic and eicosapentaenoic acids. After 48 h of enrichment, the arachidonic acid content of the fed nauplii was significantly ($P<0.05$) higher than the unfed nauplii. Eicosapentaenoic acid, however, was significantly ($P<0.05$) higher only in the nauplii fed liposomes containing dietary supplements.

Table 1. Distribution of radioactivity (dpm) among lipid classes of *Artemia* fed ^{14}C-glycine and ^{14}C-phoshatidylcholine labeled liposomes 24 and 48 h after feeding. Percentages are given in parentheses. Values are means of triplicates ± standard error. PL:phospholipids, TAG:triacylglycerides, CHOL:cholesterol, DAG:diacylglycerides, FFA:free fatty acids, WE:wax esters, CE:cholesterol esters. Superscripts indicate significant difference ($P<0.05$) between 24 and 48 h.

	^{14}C-Glycine		^{14}C-Phosphatidylcholine	
	24 h	48 h	24 h	48 h
PL	$1241^a \pm 180$ $(100^a \pm 0.0)$	$1328^a \pm 399$ $(62.1^b \pm 15.9)$	$19723^a \pm 54$ $(43.3^a \pm 3.4)$	$13165^b \pm 996$ $(42.9^a \pm 3.4)$
TAG	- -	809 ± 399 (37.9 ± 15.9)	$24830^a \pm 2752$ $(53.3^a \pm 2.8)$	$14243^b \pm 763$ $(46.5^a \pm 1.6)$
CHOL/DAG	- -	- -	$338^a \pm 24$ $(0.7^a \pm 0.4)$	$314^a \pm 17$ $(1.0^b \pm 0.0)$
FFA	- -	- -	$897^a \pm 107$ $(1.9^a \pm 0.2)$	$1766^b \pm 318$ $(5.9^b \pm 1.4)$
WE/CE	- -	- -	$358^a \pm 210$ $(0.7^a \pm 0.5)$	$1084^b \pm 404$ $(3.7^b \pm 1.7)$

Table 2. Protein, total lipid and lipid class composition (µg/mg wet weight) of *Artemia* starved and fed non-labeled liposomes for 24 and 48 h. TL: total lipid, PL:phospholipids, TAG:triacylglycerides, CHOL:cholesterol, WE:wax esters, CE:cholesterol esters. Values are means of triplicates ± standard error. Differing superscripts indicate significant difference ($P<0.05$) among values in the same row. L+D= Liposomes containing saline solution with BSA, glucose, vitamin and mineral mix, L-D= Liposomes containing only saline solution

	0 hr	24 h			48 h		
		L+D	L-D	UNFED L+D	L-D	UNFED	
Protein	$74.2^{a} \pm 3.8$	$60.2^{b} \pm 1.9$	$55.7^{c} \pm 0.9$	$52.4^{c} \pm 4.1$	$50.6^{c} \pm 3.8$	$46.8^{c} \pm 1.5$	$48.6^{c} \pm 1.2$
TL	$41.0^{a} \pm 1.2$	$25.5^{b} \pm 0.7$	$23.2^{b} \pm 1.7$	$13.8^{c} \pm 1.0$	$17.2^{c} \pm 0.4$	$15.1^{c} \pm 1.0$	$14.9^{c} \pm 1.7$
PL	$14.3^{a} \pm 1.7$	$10.7^{b} \pm 1.5$	$9.8^{b} \pm 0.9$	$7.9^{c} \pm 0.5$	$9.3^{b} \pm 0.6$	$8.6^{b} \pm 0.4$	$11.3^{b} \pm 1.0$
TAG	$21.8^{a} \pm 0.3$	$11.0^{b} \pm 0.9$	$9.8^{b} \pm 1.5$	$4.6^{c} \pm 0.5$	$3.8^{c} \pm 0.5$	$3.0^{c} \pm 0.5$	$1.9^{c} \pm 0.5$
CHOL	$2.8^{a} \pm 0.3$	$2.7^{a} \pm 0.3$	$2.6^{a} \pm 0.1$	$0.9^{b} \pm 0.1$	$3.1^{a} \pm 0.1$	$2.7^{a} \pm 0.3$	$1.4^{b} \pm 0.2$
WE/CE	$2.0^{a} \pm 0.4$	$1.1^{a} \pm 0.3$	$1.0^{a} \pm 0.2$	$0.5^{b} \pm 0.2$	$1.0^{a} \pm 0.2$	$0.8^{a} \pm 0.4$	$0.3^{b} \pm 0.2$

Table 3. Fatty acid composition (percent of total FAMEs) of liposomes and *Artemia* starved and fed non-labeled liposomes for 48 hours. Values are means of triplicates. Differing superscripts indicate significant differences ($P<0.05$) among the values in the same row. L+D= Liposomes containing saline solution with BSA, glucose, vitamin and mineral mix, L-D= Liposomes containing only saline solution

Fatty acids	0 hr	24 h			48 h			
		L+D	L-D	UNFED	L+D	L-D	UNFED	LIPOSOME
16:0	11.7	14.5	14.8	12.4	14.2	13.4	13.3	35.5
16:1n-7	4.5	4.2	3.5	4.1	2.7	2.5	2.6	1.8
17:0	0.7	0.5	0.4	0.5	0.6	0.5	0.6	0.2
18:0	4.6	8.0	8.0	7.1	9.7	9.6	9.7	12.6
18:1n-9	21.2	26.4	26.3	25.5	28.6	29.7	30.9	28.3
18:2n-6	6.4	7.9	7.4	5.1	6.1	6.2	4.7	13.4
18:3n-3	33.4	27.3	28.1	32.6	26.5	26.6	26.0	-
18:4n-3	6.8	4.6	4.9	4.6	3.8	4.4	5.6	-
20:0	0.4	-	-	-	-	-	-	-
20:1n-11	0.1	-	-	-	-	-	-	-
20:4n-6	1.5^{a}	2.4^{b}	2.5^{b}	1.6^{a}	3.3^{c}	3.5^{c}	1.6^{a}	2.9^{bc}
20:5n-3	1.6^{a}	1.9^{a}	1.6^{a}	1.4^{a}	2.7^{b}	1.7^{a}	2.0^{a}	1.8^{a}
22:6n-3	-	tr	tr	-	tr	tr	-	0.5
Unidentified	7.1	2.3	2.5	5.1	1.8	2.0	3.0	2.0

Figure 1. Uptake (%) of ^{14}C labeled liposomes by *Artemia* nauplii after 24 and 48 hours. Values are means ± s.e. of triplicate measurements. * indicates a significant difference ($P<0.05$) between 24 and 48 h. PC; phosphatidylcholine labeled liposomes, GLY; glycine labeled liposomes. Numbers above bars indicate total counts (dpm).

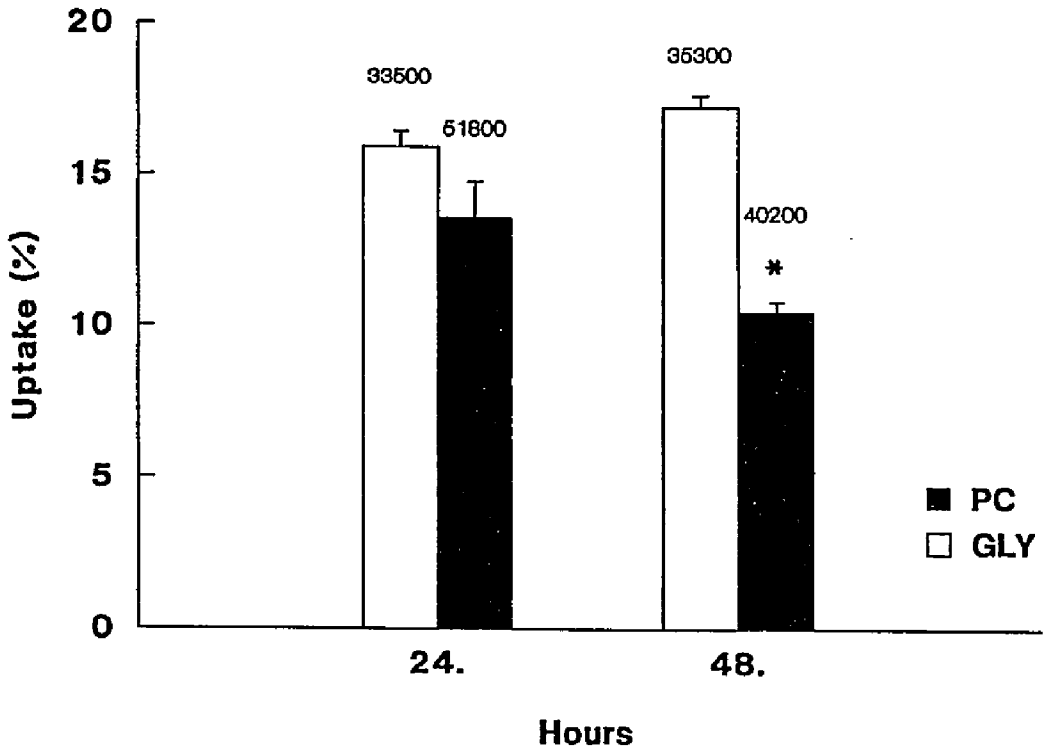
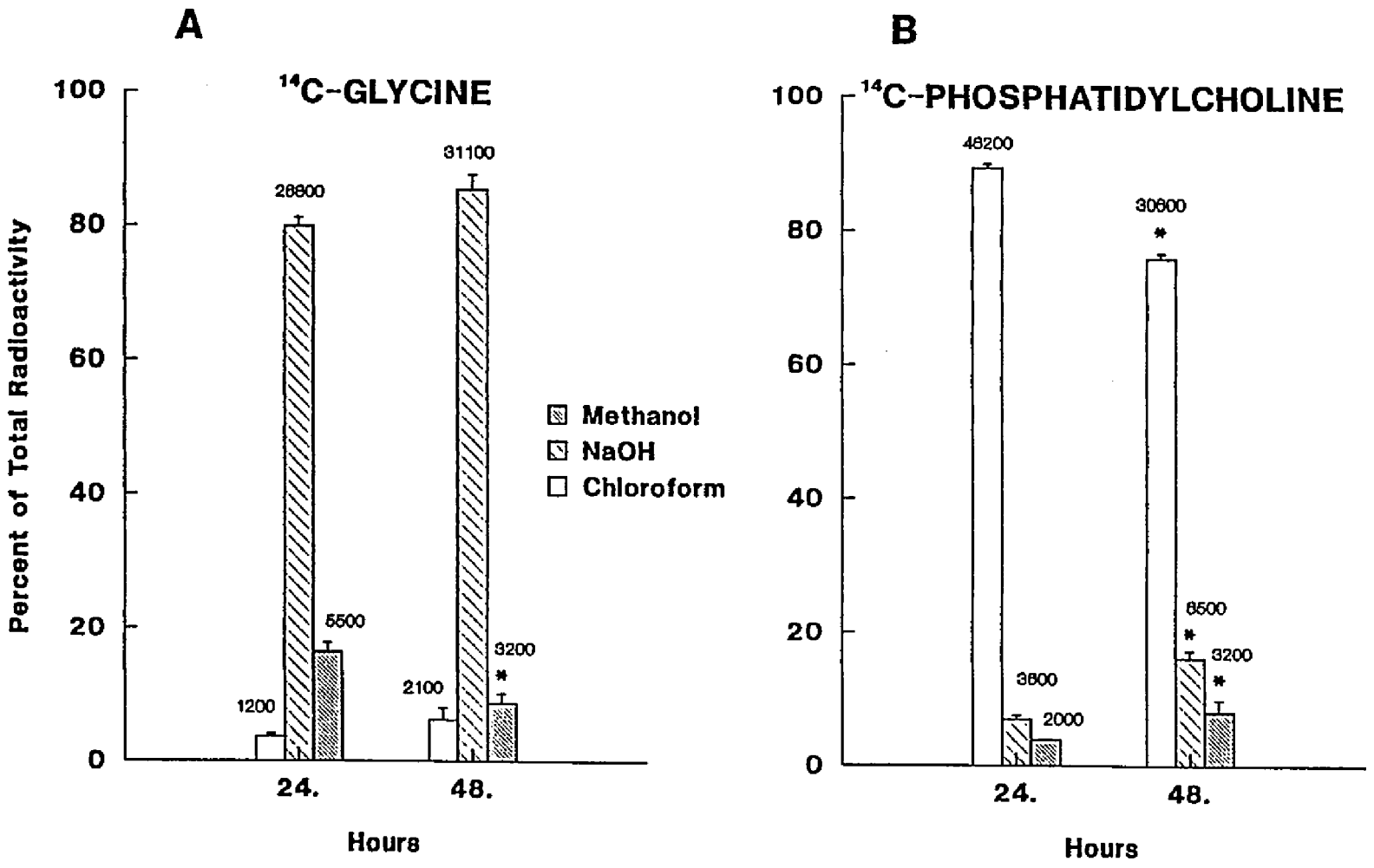

20
15
10
5
0
Uptake (%)
33500
51600
35300
40200
*
PC
GLY
24.
48.
Hours

Figure 2. Percent distribution of ^{14}C activity in chloroform, methanol fractions and NaOH hydrolysate of *Artemia* nauplii fed (**A**) ^{14}C-Glycine and (**B**) ^{14}C-Phosphatidylcholine labeled liposomes for 48 h. Values are means ± s.e. of triplicate measurements. * indicates a significant difference ($P<0.05$) between 24 and 48 hours. Numbers above bars indicate total counts (dpm).


A
^{14}C-GLYCINE
Percent of Total Radioactivity
100
80
60
40
20
0
1200
28600
5500
2100
31100
3200
*
24.
48.
Hours
Methanol
NaOH
Chloroform
B
^{14}C-PHOSPHATIDYLCHOLINE
46200
3600
2000
30600
*
6500
*
3200
*
24.
48.
Hours

Discussion

The results of the present study suggest that liposomes were readily ingested and assimilated by *Artemia* nauplii within 24 hours of hatching. Incorporation of a significant proportion of ^{14}C-glycine label in NaOH hydrolysate indicated that the liposomes fed to the nauplii were taken up and metabolized, rather than adsorbed onto the exoskeleton of the nauplii. Relatively low uptake (15%) of label by the nauplii may be due to the smaller size of liposomes. Parker and Selivonchick (1986) showed that radio-labeled PC, glucose and amino acids were effectively absorbed from liposomes by *Crassostrea gigas* juveniles with the total tissue radioactivity representing 10 % of the total administered label. In this study, 15 % of the total radioactivity from both ^{14}C-glycine and ^{14}C-PC was recovered in the nauplii after 24 h of enrichment. Furthermore, incorporation of ^{14}C-PC into TAG and other lipid classes suggests that phospholipid membranes of liposomes were possibly broken down by the action of phospholipase A_2. It has been demonstrated that *Artemia* nauplii possess major lipolytic enzymes such as triacylglycerol lipase, phospholipase A_2 (Ozkizilcik et al. 1994) and acid hydrolases (Perona and Vallejo, 1985). The significant drop in total ^{14}C-PC incorporation after 48 h was possibly due to the high catabolic demand of development on lipids. We did

not measure the $^{14}CO_2$ evolution which may account for the drop of total activity after 48 h.

Biochemistry of embryogenesis in *Artemia* appears to be a complex process involving simultaneous synthesis and catabolism of lipids. The high energetic demand of development is clearly reflected in the lipids where a significant proportion of energy is stored. Benijts et al. (1975) reported a 26% decrease in the total lipid content of *Artemia* during the first naupliar stages. In a separate study, Navarro et al. (1991) showed a significant decrease of TAG between the nauplii and metanauplii stages while polar lipid remained unchanged. In the present study, a much greater decrease (66%) was observed in total lipids of unfed nauplii. In liposome fed nauplii, this decrease was less dramatic (37%). An even distribution of ^{14}C-PC activity between polar and neutral lipids of 24 h old *Artemia* suggests a rapid turnover of fatty acyl moieties among lipid classes. Furthermore, incorporation of ^{14}C-glycine into phospholipids suggested that the carbon skeleton from ^{14}C-glycine may be used in *de novo* synthesis of phospholipids indicating their role in membrane synthesis. Moreover, respired $^{14}CO_2$ may be taken up from the culture medium.

Lasic (1992) has reviewed the methods of incorporating

various molecules in liposomes. Hydrophilic compounds can be dissolved in the aqueous core of liposomes, while lipophilic compounds can be incorporated into the lamellae bilayer. Alternatively, some larger molecules such as proteins can be conjugated to the phospholipid head group, especially to those containing amino groups, through electrostatic and hydrophobic binding (Sweet and Zull, 1970). Recent studies have demonstrated the feasibility of live food mediated drug delivery for disease treatment in larviculture (Verpraet et al., 1992; Dixon et al. 1994). Verpraet et al. (1992) have successfully bioencapsulated trimethoprim and sulfamethaxazole in *Artemia* and rotifers. In a separate study, Dixon et al. (1994) have shown that the antibacterial drug sarafloxacin was rapidly taken up by *Artemia*, and could be detected after two hours of enrichment. Both studies, however, used highly hydrophobic drugs emulsified within a commercial enrichment diet. In this study, the uptake of ^{14}C-glycine (Fig. 1) clearly showed that hydrophilic nutrients and drugs could be bioencapsulated using liposomes. However, the time required for enrichment should be determined carefully to avoid the metabolism of drugs by the nauplii to less active forms.

Typically, newly hatched nauplii are kept in culture water for 24 h prior to enrichment (Leger et al., 1986). During this period, the size of nauplii increases, thus

making them unsuitable prey items for most fish larvae at first feeding. Moreover, total lipid and protein content of nauplii decrease significantly, thus, reducing their nutritional quality (Navarro et al., 1991). In the present study, *Artemia* nauplii were enriched immediately after hatching. Total lipid content of the nauplii fed liposomes was found to be two fold higher than that of the unfed nauplii (Table 2). In this context, we propose that liposomes containing high levels of phospholipids of marine origin could serve as an effective way to enrich *Artemia* with n-3 PUFA. In the present study, however, bulk PC used in the preparation of liposomes contained only small amounts of n-3 PUFA. Therefore, no remarkable change was observed in fatty acid composition of the nauplii fed liposomes, except for arachidonic and eicosapentaenoic acids. Moreover, the incorporation of 17 % of ^{14}C-glycine in the methanol fraction suggested that liposomes could be used to elevate the total free amino acid content of the nauplii as well as to enrich nauplii with essential amino acid. Teshima et al. (1986) reported higher growth rates of *Panaeus japonicus* larvae when the purified diets were supplemented with free amino acids.

In conclusion, the results presented in this study suggest that liposomes emerge as a new method to enrich live diets with phospholipids and free amino acids and possibly

bioencapsulate therapeutic drugs for marine fish larvae.

CHAPTER 4

ONTOGENETIC CHANGES OF LIPOLYTIC ENZYMES IN STRIPED BASS (*MORONE SAXATILIS*)

Introduction

Lipids play a central role in the growth and development of marine fish larvae. Lipids stored in the eggs provide a considerable amount of energy and polyunsaturated fatty acids for the synthesis of membranes and bioactive compounds, such as eicosanoids (Sargent et al., 1989) until exogenous feeding is established (Fraser et al., 1988). Marine fish larvae prey primarily on lipid-rich zooplankton (e.g. copepods) in their early stages of development. The lipid composition of zooplankton, however, varies considerably depending on species, season, geographical location and nutritional status (Lee et al., 1972). Triacylglycerols, wax esters and phospholipids together make up over 90% of the total lipids (Lee, 1974).

Eggs and the early larval stages of striped bass are characterized by a large distinctive oil globule that forms nearly 55% of the body mass and contains >90% wax esters (Eldridge et al., 1983). Striped bass larvae do not utilize their lipid reserves upon starvation and most unfed larvae die with an intact oil globule (Eldridge et al. 1982). This rare observation was also confirmed in our unpublished studies and led us to hypothesize that striped bass larvae may lack lipolytic enzymes and thus, the initial digestive processes are facilitated by the exogenous enzymes present

in live zooplankton (Dabrowski, 1979; Lauff and Hofer, 1984).

Thus far, studies on the digestion and absorption of dietary lipids have been conducted exclusively on juvenile and adult fish species (Patton and Benson, 1975; Patton et al., 1975; Tocher and Sargent, 1984). The digestive system of premetamorphosed fish larvae, however, is morphologically, anatomically and physiologically different from that of adults (Govoni, 1986). Proteases and amylases have been studied in several species of fish larvae (Baragi and Lovell, 1986; Kawaii and Ikeda, 1973; Lauff and Hofer, 1984), however, very little information exists on the lipolytic enzymes in fish larvae and the widely used live food *Artemia*. In this study, I investigated the ontogenetic changes of three major lipolytic activities, namely, triacylglycerol hydrolase (TAGH), wax ester hydrolase (WEH) and phospholipase A_2 (PLA_2), in the fertilized eggs and premetamorphosed larvae of striped bass and the live food *Artemia*.

Materials and methods

Radiolabels and chemicals

Glyceryl tri-[1-^{14}C]oleate (110 mCi/mmol), oleic acid [1-^{14}C] (56 mCi/mmol) and phosphatidylcholine L-α-dipalmitoyl-[2-palmitoyl-1-^{14}C] (55.5 mCi/mmol) were purchased from NEN

Research Products (Boston, MA). Triolein, phosphatidylcholine, palmityl alcohol and palmityl oleate were obtained from Sigma Chemical (St. Louis, MO). The A grade sodium salts of glycochenodeoxycholate, taurodeoxycholate, taurochenodeoxycholate (TCDC), and taurocholate (TC) were purchased from Calbiochem (La Jolla, CA). Hydroxysteroid dehydrogenase (Grade II) was purchased from Sigma Chemical (St. Louis, MO). All other chemicals were reagent grade and the solvents were HPLC grade (Baxter Scientific Products Co., Raleigh, NC). Whatman 3-MM chromatographic filter paper was obtained from VWR Scientific (Philadelphia, PA).

Fertilized eggs and larval fish

Fertilized eggs and post-hatching larvae were obtained from King and Queen State Hatchery, Virginia and transferred to the laboratory in a container filled with culture water (3 ppt). Three replicates of 20 eggs or 20 larvae each were weighed and stored frozen the same day at -80°C for enzyme assays. Six days post-hatching (DPH) larvae were transferred to three conical tanks (70 liter, 2,000 larvae/tank) in a recirculating system. Culture water (21 °C, 3 ppt) was passed through a 5 μm filter and crushed oyster shells to eliminate uneaten food items, fecal particles and ammonia. Feeding was initiated on seven DPH using San Francisco Bay origin *Artemia* nauplii, and a food

density of 5 nauplii/ml was maintained by periodically counting the number of nauplii in the culture water throughout the experiment. The *Artemia* cysts were hatched daily in 60 L transparent tanks in 1 µm filtered estuarine water (24 ppt) at 28 °C. Freshly hatched nauplii were collected on 150 µm filters, rinsed well and stored at 10 °C for feeding.

Preparation of enzyme extract

The fed larvae were sampled 12-14 hours after last feeding and examined under a light microscope for the presence of *Artemia* nauplii in their digestive tract. Eggs and larvae (20/sample) and *Artemia* nauplii (approximately 150 mg wet weight/sample) were homogenized (Ultra-turrax, IKA Works Inc., Cincinnati, OH) in 1 ml of ice-cold buffer containing 50 mM Tris-HCl, 100 mM NaCl and 40 mM $CaCl_2$ at pH 8.5 for TAGH and WEH assays. Phospholipase A_2 was extracted in an identical manner in 1 ml of ice-cold buffer containing 0.1 M Tris-HCl, 8 mM $CaCl_2$, 0.1% (v/v) Triton X-100® at pH 8.0. The homogenates were then centrifuged for 30 min at 20,000 *g* (Eppendorf Microcentrifuge) at 4 °C. Supernatants were collected in a clean microfuge tube, stored at -80 °C overnight and assayed the following day.

Synthesis of palmityl [1-^{14}C]oleate

Labeled palmityl oleate was synthesized from palmityl

alcohol and oleic acid (labeled and non-labeled) according to Place and Roby (1986). Fifty five µmol cold and labeled (25 µCi [1-^{14}C]) oleic acid was dissolved in toluene in a glass test tube. A molar equivalent of palmityl alcohol and 0.1 molar equivalent of p-toluenesulfonic acid was added to the mixture and incubated at 85 °C for two hours. After cooling to room temperature, the reaction was stopped by adding nine ml of petroleum ether. The labeled palmityl oleate was purified on a 10 cm^3 silicic acid column by eluting with three column volumes of 2 % (v/v) diethyl ether in petroleum ether. The purified palmityl ^{14}C-oleate was dried under nitrogen and stored at -20 °C in 500 µl of toluene. Radio and chemical purities were examined by thin layer chromatography on HPTLC (Whatman silica gel G) plates after developing in hexane:diethyl ether:acetic acid (80:20:2 by volume). The radiopurity of palmityl (^{14}C)oleate was measured by liquid-scintillation counting of TLC scrapings and determined to be 98.9 ± 0.3 %. Chemical purity of the label was determined to be 99+% by using photodensitometry (Hoefer Scientific Instruments, San Francisco, CA) after charring the plates with cupric acetate reagent (Fewster et al 1969).

Preparation of substrate suspensions

Triolein [carboxyl-1-^{14}C], palmityl [1-^{14}C]oleate and phosphatidylcholine [sn-2-1-^{14}C] were used for the assays of

TAGH, WEH and PLA_2, respectively. Stock solutions of TAGH and WEH substrates were prepared from labeled and non-labeled triolein and palmityl oleate in warm ethanol (40 °C). Known quantities of stock solutions were added into a buffer containing 5% (w/v) fatty acid free bovine serum albumin, 100 mM potassium phosphate, 10 mM EDTA, at pH 7.4 and stirred vigorously (Tocher and Sargent, 1984). The suspensions were stored at 4 °C and stirred for 5 min before use. For the PLA_2 assay, known quantities of labeled and non-labeled phosphatidylcholine (PC) were dissolved in chloroform:methanol (1:1 v/v). Solvent was evaporated under a stream of nitrogen. A buffer containing 0.1 M Tris-HCl, 8 mM $CaCl_2$, 0.1% Triton X-100^R at pH 8.0 was added to the substrate (Neas and Hazel 1985). The suspension was sonicated for 2 min and shaken vigorously. The final substrate concentration of all the enzyme assays was 500 µM. For TAGH and WEH sodium taurocholate (5 mM) was added to the substrate suspensions, since this was the dominant bile salt in the bile of young striped bass. Approximately 0.5 to 1 % of the substrate was hydrolyzed in the assays.

Enzyme assays

One hundred µl of substrate suspension was added to 100 µl of enzyme extract and incubated at 21 °C for 4 h. Fatty acid released was linear with time up to 8 h. R^2 values for TAGH, WEH and PLA_2 were 98, 97 and 86, respectively. Blanks

containing no enzyme were run in parallel to determine the autolysis of the substrates. The enzyme activities were linear with the protein content of the enzyme extracts. The reactions were stopped by the addition of 750 µl of chloroform, methanol and toluene (2:2.4:1, v/v/v) containing 0.3 mM stearic acid, and 25 µl of 1 N NaOH (Khoo and Steinberg, 1975). The solution was stirred on a vortex mixer and centrifuged for 5 min at 20,000 *g*. Extraction efficiencies, as determined by TLC/FID, were 97, 94 and 95 % for TAGH, WEH and PLA_2, respectively. A three hundred µl aliquot of the upper phase was transferred into a scintillation vial and 5 ml of scintillation cocktail added (Ecolite +, ICN Biomedicals, Costa Mesa, CA). Radioactivity in the samples was counted using a Beckman LS5000TD liquid scintillation counter and corrected for the aliquot size and the fatty acid moieties of the substrate. Results were calculated from specific activities of each substrate and expressed as pmol fatty acid liberated/hr/egg or larva or mg protein. Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Dietary contribution of lipolytic enzymes was estimated from average stomach contents (Eldridge et al. 1982) and individual dry weight (1.8 µg) of *Artemia* nauplii (Leger et al. 1986) as given below:

(1) **mg ww *Artemia* in full gut =**

$$\frac{\text{\# of } \textit{Artemia} \text{ in full gut} \times \text{mean dw of } \textit{Artemia}}{[100 - \text{moisture content (80 \%)}]}$$

(2) **Enzyme Activity of *Artemia* in full gut =**

Activity of *Artemia* (pmol/hr/mg ww) x mg ww *Artemia* in full gut

$$\text{(3) Contribution} = \frac{\text{Enzyme activity of } \textit{Artemia} \text{ in full gut}}{\text{Activity of } (\textit{Artemia} + \text{Larva})}$$

Bile analysis

Ten 1.5 year old striped bass (150 ± 12.0 g) were killed by decapitation. Bile was removed from the gall bladder with a sterile 1 cc tuberculin syringe and placed in a tared sterile 0.5 ml polyethylene centrifuge tube. An equal volume of methanol was added and the precipitated proteins removed by centrifugation. Total bile salts were assayed with 3 α-hydroxysteroid dehydrogenase (EC 1.1.150) (Coleman et al. 1979). Whole bile aliquots were analyzed by HPLC on Waters Nova 5 μ Radial packs using a linear gradient (1.2 to 34 min, flow rate 2.8 ml/min) from initial conditions (3.21 mM phosphoric acid, 3.75 mM KOH, and 4.0 mM KH_2PO_4, pH 4.32, 68:32 (v/v) methanol:water) to a final condition (3.21 mM phosphoric acid, 3.75 mM KOH and 11 mM KH_2PO_4, pH 4.32, 68:32 (v/v) methanol:water). Bile salts were detected and quantitated by their abrosbance at 204 nm in comparison with standards. To assay biliary lipids aliquots of the

methanolic bile were spotted on Chromarods-SII and developed sequentially in the three solvent system described by Honkanen et al. (1985). Determination of bile salt mass was preformed by comparison with a standard curve generated for concentrations of TCDC and TC between 1 and 20 µg. Operating conditions for the Iatroscan TH-10 analyzer (Iatron Laboratories, Tokyo, Japan) were the same as those described by Rigler et al. (34). Samples were spotted on type SII chromarods (Iatron Laboratories) which were activated by scanning through a hydrogen flame twice. Integration was performed by Hewlett-Packard 3390-A integrator (Avondale, PA). Chromarods were developed in equilibrated filter paper-lined glass tanks containing 75 ml of solvent. When multiple systems were used, chromarods were dried according to the method of Harvey and Patton (1981).

Statistical analysis

Two-way ANOVA was employed to determine the effects of larval age (days post-feeding) and feeding followed by pairwise multiple comparison of means (Tukey) using the Systat^{R} statistical package. All data were $\log_{10}$ transformed before analysis.

Results

Fig.1 shows the effect of substrate and Taurocholate

concentrations on lipolytic activities. The activities of TAGH, WEH and PLA_2 increased with increasing substrate concentrations. The maximum activity for each enzyme was around 500 μM substrate concentration, above which the activities either decreased (PLA_2) or remained the same (TAGH and WEH). Addition of bile salt, TC, to the assay medium significantly increased the activities of TAGH and WEH (Fig.1 B). The maximum activities of both TAGH and WEH were at 5 mM TC. Therefore, this concentration was used later for TAGH and WEH assays. PLA_2 was excluded from bile salt assays, since the assay medium for PLA_2 contained 0.1 % non-ionic detergent Triton X-100^R.

Fertilized eggs of striped bass showed low activities of TAGH (16 pmol/hr/egg), WEH (16 pmol/hr/egg) and PLA_2 (25 pmol/hr/egg) which remained constant until 5-6 DPH at which time a slight increase was observed (Fig.2 A,B,C). PLA_2 was the dominant activity and showed a steady increase following hatching. With the onset of feeding, all three enzymes increased significantly ($P<0.05$). TAGH activity was significantly higher ($P<0.05$) in fed larvae than unfed larvae and increased 18-fold to 903 pmol/hr/larva after 13 days of feeding. WEH demonstrated a trend similar to TAGH, with the exception that there was no significant difference between fed and unfed larvae at 12 DPH. Despite initial high levels, PLA_2 did not increase as drastically as TAGH

and WEH after feeding. At both 16 and 20 DPH, PLA_2 activity of the fed larvae increased significantly ($P<0.05$) and was higher than the unfed larvae ($P<0.05$). The 12 DPH unfed larvae, however, had significantly higher ($P<0.05$) PLA_2 activity than the fed larvae.

Specific activities (pmol/hr/mg protein) of TAGH, WEH and PLA_2 are shown in Fig.2 D,E,F. Total protein content of the enzyme extracts determined the outcome of the specific activities. Fed larvae had significantly higher ($P<0.05$) amounts of total protein than the unfed larvae (Fig 3). The effect was quite apparent with WEH and PLA_2 and less so for TAGH. Although the general trend indicated an increase of WEH in both fed and unfed larvae, there were no significant differences between groups based on specific activity. Specific activity of PLA_2 was significantly higher in unfed larvae than that of the fed larvae. TAGH, on the other hand, illustrated a similar pattern to that seen in absolute activities (pmol/hr/egg-larva).

The relative changes of TAGH, WEH and PLA_2 during the development of eggs and larvae are shown in Table 1. In fertilized eggs, the activities were similar. At the beginning of exogenous feeding the proportion was 3.6:6.4:1, with PLA_2 being the dominant enzyme. At 20 DPH, however, TAGH activity of the fed larvae was approximately six times

that of the WEH and three times that of PLA_2.

Lipolytic enzymes of live food *Artemia* were also measured to determine the exogenous enzyme contribution. PLA_2 activity was measured in decapsulated cysts of *Artemia*, while TAGH and WEH activities were undetectable (Fig. 4). In *Artemia*, all three enzymes increased steadily during and after hatching. Contribution of dietary enzymes from *Artemia* to the total lipolytic process was calculated at 7, 12, 16 and 20 DPH fed larvae (Table 1). The contribution of exogenous enzymes to the total lipolytic process in developing striped bass larvae ranged from 0.1% to 5.8%. PLA_2 had the highest contribution followed by TAGH and WEH.

The gall bladder bile salt concentration in young immature striped bass was found to be 209.6 ± 27.1 (sd) mM by the enzymatic hydroxysteroid dehydrogenase procedure and 230 ± 20.5 (sd) mM by the Iatroscan procedure. Only two bile salts were found, taurocholate and taurochenodeoxycholate with taurocholate being the dominant (>85%) bile salt. The biliary phospholipid content was 1.69 ± 0.79 mM and the cholesterol content was 3.01 ± 1.01 mM.

Table 1. Proportions of TAGH, PLA_2 and WEH activities (WEH taken as base value) and the contribution (% of total) of digestive enzymes from *Artemia*.

	TAGH:PLA_2:WEH		
Days	pmol/hr/Egg-Larva	pmol/hr/mg protein	% Contribution from *Artemia*
Post-fertilization			
1	1.1 : 1.6 : 1	0.8 : 1.4 : 1	-
2	2.7 : 7.4 : 1	2.0 : 7.4 : 1	-
3	0.9 : 2.6 : 1	0.5 : 2.1 : 1	-
Post-hatching			
1	1.0 : 3.8 : 1	0.4 : 1.8 : 1	-
2	1.3 : 4.6 : 1	0.7 : 3.3 : 1	-
3	1.7 : 6.3 : 1	2.4 : 15.1 : 1	-
4	2.1 : 13.7 : 1	1.7 : 11.5 : 1	-
5	4.6 : 7.0 : 1	2.8 : 5.5 : 1	-
6	4.7 : 7.0 : 1	2.8 : 5.2 : 1	-
7*	3.6 : 6.4 : 1	2.3 : 4.0 : 1	1.1 / 1.7 / 0.1
12 (Fed)	4.7 : 5.8 : 1	3.8 : 3.4 : 1	1.1 / 2.5 / 0.1
12 (Unfed)	1.5 : 8.0 : 1	1.0 : 5.9 : 1	-
16 (Fed)	4.9 : 2.9 : 1	3.7 : 2.1 : 1	0.5 / 2.3 / 0.1
16 (Unfed)	2.5 : 9.0 : 1	1.4 : 5.8 : 1	-
20 (Fed)	6.3 : 1.9 : 1	5.2 : 1.3 : 1	0.6 / 5.8 / 0.1
20 (Unfed)	3.8 : 2.4 : 1	2.3 : 2.2 : 1	-

* Initiation of feeding

Fig.1 Effect of (**A**) substrate concentration on the activities of TAGH, WEH and PLA_2 (inset) and (**B**) Taurocholate concentration on the activities of TAGH and WEH (pmol fatty acid released/hr/mg protein). Assay conditions were as described in Material and Methods. Enzymes were extracted from 20 days post-hatching striped bass larvae. Substrate assays contained 5 mM Taurocholate. Bile salt assays were performed with 500 µM substrate concentration. Results are means (N=3) ± se.

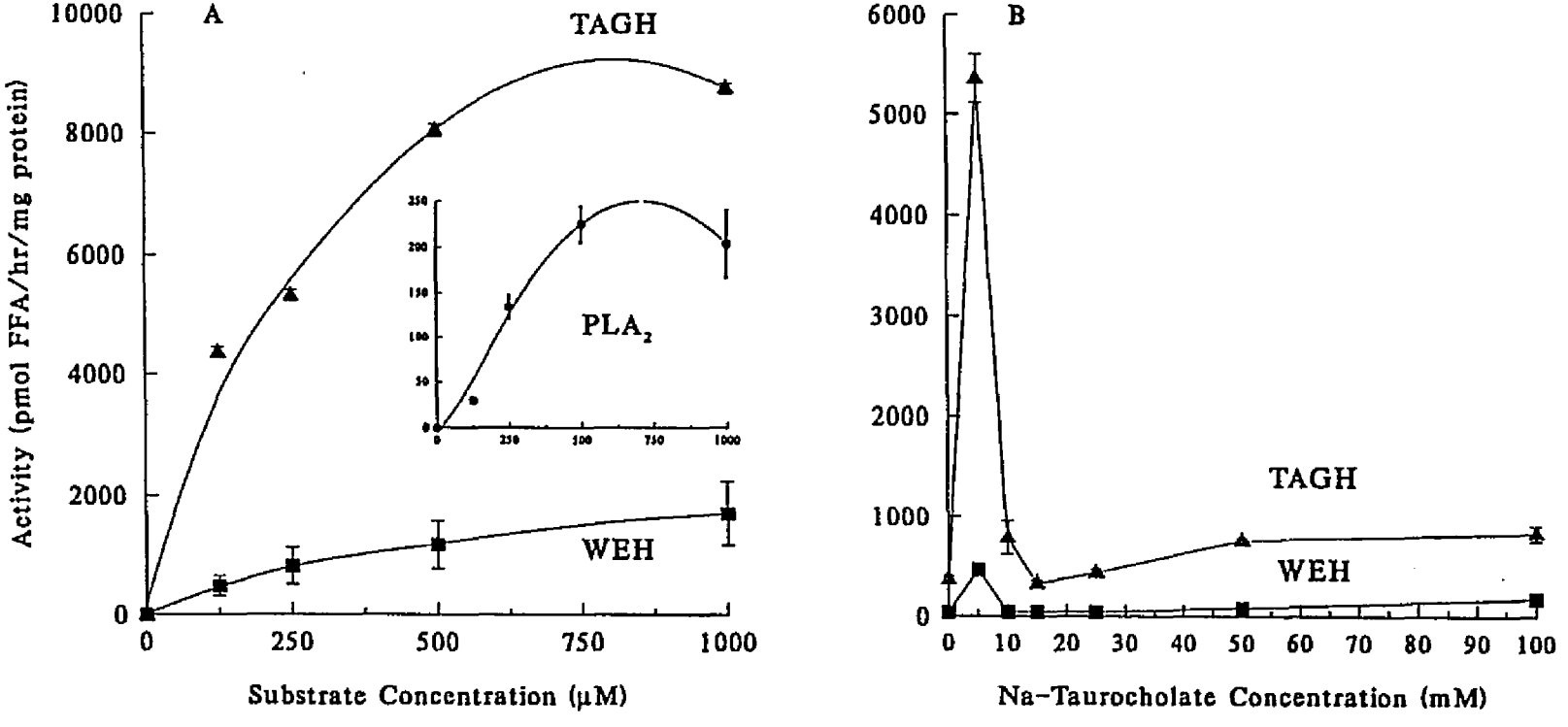

A
TAGH
WEH
PLA2
Activity (pmol FFA/hr/mg protein)
Substrate Concentration (μM)
10000
8000
6000
4000
2000
0
0
250
500
750
1000
B
TAGH
WEH
Na-Taurocholate Concentration (mM)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
100

Fig.2 TAGH, WEH and PLA_2 activities in fertilized eggs, and fed (closed symbols) and unfed (open symbols) larvae of striped bass during development. Figs **A, B,** and **C** represent absolute activities (pmol Fatty Acid/hr/Egg or Larva). Figs **D, E,** and **F** represent specific activities (pmol Fatty Acid/hr/mg protein). Values are means (N=3) ± se. Lower case letters indicate significant difference ($P<0.05$). H, hatching; F, start of feeding.

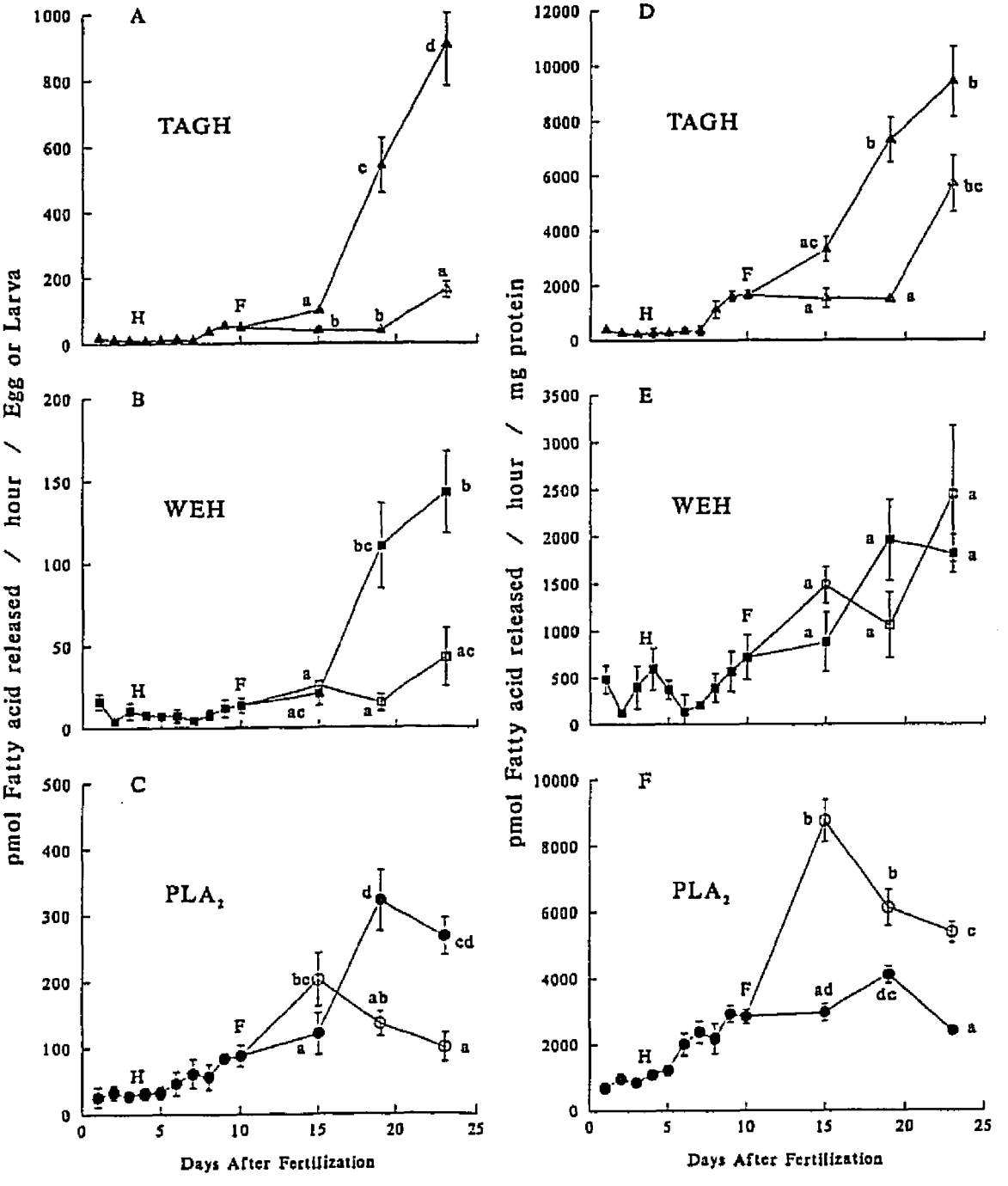

A
TAGH
B
WEH
C
PLA2
D
TAGH
E
WEH
F
PLA2
pmol Fatty acid released / hour / Egg or Larva
pmol Fatty acid released / hour / mg protein
Days After Fertilization
Days After Fertilization

Fig.3 Total soluble protein content of fertilized eggs, and fed (closed symbols) and unfed (open symbols) larvae of striped bass during development. Values are means (N=3) ± se. H, hatching; F, start of feeding.

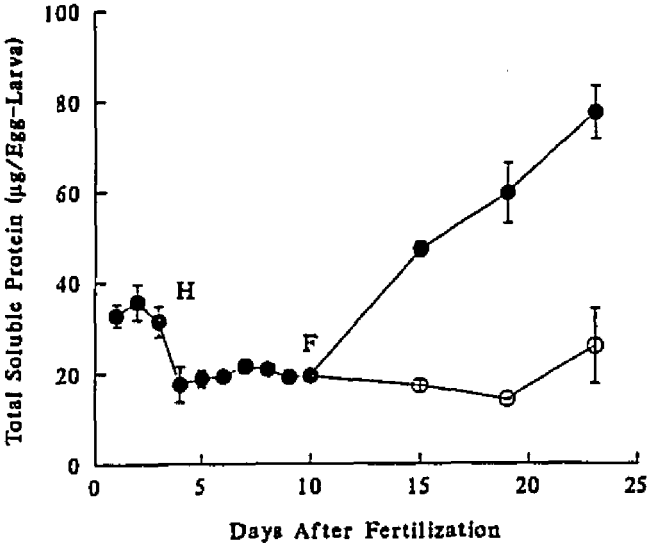
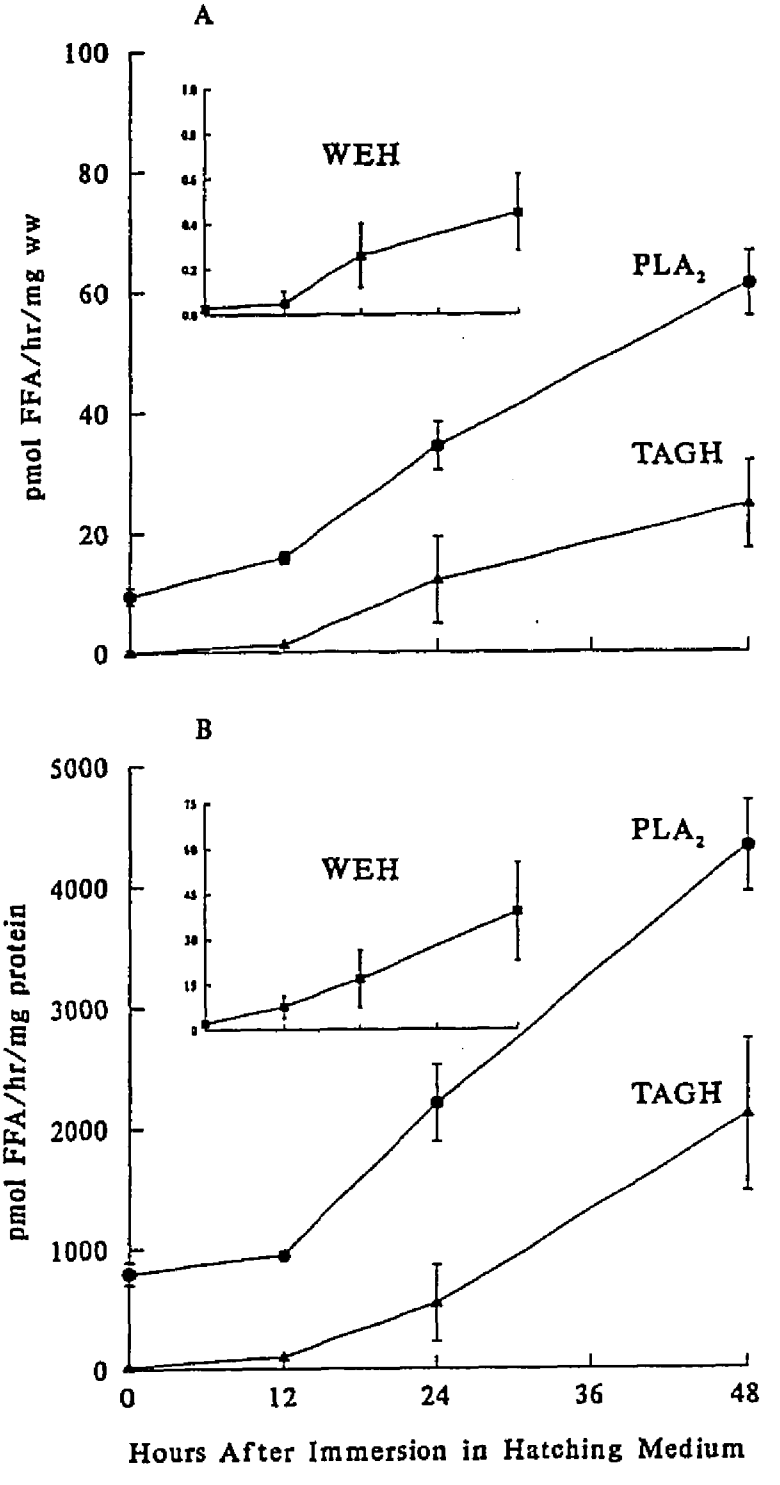

Total Soluble Protein (μg/Egg-Larva)
100
80
60
40
20
0
H
F
0
5
10
15
20
25
Days After Fertilization

Fig.4 TAGH, WEH (insets) and PLA_2 activities (**A** pmol fatty acid/hr/mg ww, **B** pmol fatty acid/hr/mg protein) in chemically decapsulated cysts (0 and 12 h) and newly hatched (24 and 48 h) nauplii of *Artemia* used as food for striped bass larvae. Values are means (N=3) ± se.


A
100
80
60
40
20
0
pmol FFA/hr/mg ww
WEH
PLA2
TAGH
B
5000
4000
3000
2000
1000
0
pmol FFA/hr/mg protein
WEH
PLA2
TAGH
0
12
24
36
48
Hours After Immersion in Hatching Medium

Discussion

In adult teleosts, the pancreas is not a discrete organ, but rather a diffuse tissue associated with the pyloric caeca. In newly hatched striped bass larvae, however, the pancreas is a separate rudimentary organ that does not diffuse into the pyloric tissues until metamorphosis (Gabaudan 1984). Preparations from pyloric caeca of trout, sardine and cod have been shown to have lipolytic activity, however, pancreatic lipase/colipase and carboxyl ester lipase have not been isolated from the same species (Leger et al. 1979; Mukundan et al. 1985; Gjellesvik et al. 1992). A study of lipase(s) of pancreatic origin in the cod (*Gadus morhua*) revealed the presence of only one pancreatic lipase; carboxyl ester lipase (Gjellesvik et al. 1992). The enzyme was a 60 kD, glycosylated protein and its activity was bile salt dependent. In the present study, the activities of TAGH and WEH were also dependent on the bile salt, TC. Above and below the critical micellar concentration (10-20 mM) the activities increase markedly, suggesting the presence of a carboxyl ester lipase-like activity. Similar results were reported by Tocher and Sargent (1984) that TAGH, WEH and sterol ester hydrolase activities were determined by bile salt concentrations of the assay mixtures.

Bile appears essential for efficient neutral lipid hydrolysis in larval striped bass. The gall bladder develops at 3 days post-hatching with bile ducts connecting the gall bladder to the ventral side of the intestine (Gabaudan, 1984). The composition of bile in fish eggs and larvae is not known. Fish bile salts are mainly trihydroxycholanic (Haslewood, 1967), consistent with the major bile salts of striped bass. As expected for a carnivorous diet (Haslewood 1967), the bile salts in striped bass are largely taurine conjugated. Interestingly, the bile of striped bass is more complex than the stomachless minnow, *Fundulus heteroclitus* (Honkanen et al. 1985), where biliary phospholipid and cholesterol are absent.

The results of this study shows that the fertilized eggs and larvae of striped bass possess the three major lipolytic enzymes, TAGH, WEH and PLA_2. It has been consistently reported that fish hydrolyze TAG much faster than WE, even though their natural diets contain significant quantities of WE (Patton et al., 1975; Patton and Benson, 1975; Mankura et al., 1984; Tocher and Sargent, 1984; Lie and Lambertsen, 1985). Our results are in agreement with these findings. The TAGH activity in striped bass larvae is up to six times higher than WEH activity. However, in rainbow trout, TAGH activity is one to two orders of magnitude greater than that of WEH activity (Tocher and Sargent, 1984). Various factors

are known to influence the efficiency of wax ester digestion and absorption in fish. Rainbow trout fed a WE rich diet showed higher levels of WEH and an increased volume of bile (Tohcer and Sargent, 1984). Bile salts transport the hydrolysis products from the site of reaction, thus, protecting lipases from potential inhibitory effects of fatty alcohols (Mattson et al., 1970; Ferreira and Patton, 1990) released from WE hydrolysis. The structure of the digestive system in fish has also been suggested to be a determining factor in the hydrolysis of dietary lipids (Patton and Benson, 1975; Place, 1992). Patton and Benson (1975) suggested that the length of time fish retained food in the pyloric caeca increased the time of exposure to the enzyme, thus insuring complete hydrolysis. In a comparative study of wax ester digestion, Place (1992) emphasized the importance of the stomach in the emulsification and retention of wax esters. The digestive system of striped bass larva, however, consists of a simple tube that is divided into anterior and posterior sections by a sphincter. Stomach and pyloric caeca do not form until metamorphosis and the gut retention time is 2.5 h (Eldridge et al., 1982). In spite of these restrictions, larva apparently have the ability to digest and absorb lipids. As of now, the physical state of dietary lipids in the digestive tract of larva is not known. Whether the WE rich diets increase the activities of WEH also needs to be studied.

Most studies on phospholipid digestion are concerned with their interaction with the digestion and absorption of other dietary lipids, and enterohepatic circulation of biliary lecithin (Thomson and Dietschy, 1981). Phospholipids make up a significant proportion of the lipids in zooplankton and contain higher levels of polyunsaturated fatty acids than TAG or WE (Lee, 1974). Besides its role in the digestion of phospholipids, PLA_2 is also responsible for the restructuring of the membranes (Neas and Hazel, 1985). Epithelial tissues of the digestive system in fish differentiate dramatically during development. The organogenesis is likely to require a high rate of membrane synthesis and turnover. Therefore, in fish larvae PLA_2 may play a dual role in the hydrolysis of dietary phospholipids and membrane restructuring in digestive tissues. Uematsu et al. (1992) reported the ontogeny of intestinal PLA_2-like proteins in the red sea bream, *Pagrus major*, using immunocytochemistry. The enzyme was absent until 13 days after hatching in the pancreatic acinar cells and the intensity of labeling increased markedly during growth. In the present study, PLA_2 is the dominant lipolytic enzyme in the eggs and unfed larvae of striped bass. PLA_2 appears to play an important role in the catabolism of phospholipids during starvation. It should be noted that our results cannot differentiate between the PLA_2 activity of the digestive system and the body mass, since the whole larva

was used for the extraction of the enzyme. Currently, very little is known about the biochemical processes involved in the digestion and absorption of dietary phospholipids in fish. Other phospholipases and diacylglycerol hydrolase may be important enzymes in phospholipid hydrolysis.

It has long been advocated that the initial digestion in larvae proceeds by the action of the enzymes present in the live food (Dabrowski, 1979; Lauff and Hofer, 1984). The results of the present study reveal that the lipid digestion in striped bass is not governed by exogenous enzymes. The contribution of dietary enzymes to the total lipolytic activity is minimal (<6.5%). It is particularly negligible for TAGH and WEH. Using the data from Eldridge et al. (1981) and Ozkizilcik and Chu (1994b), we estimate that 7 DPH larva consumes approximately 2.6 µg TAG, 0.2 µg WE and 1.7 µg phospholipids daily . The larva possesses a lipolytic capacity to digest 14, 71 and 94 % of the dietary TAG, WE and phospholipids, respectively. Nevertheless, the exogenous enzymes from the live diet may secondarily affect the digestion of lipids. Small amounts of monoglycerides, protonated fatty acids along with phospholipids may enhance emulsification of lipid droplets and form stable aggregates that are the physiological substrates for lipases (Carey et al., 1983). This may be an important step in the emulsification of dietary lipids in the larva that lacks a

stomach. Furthermore, synthesis and secretion of digestive enzymes in fish larvae may be triggered by hormone-like compounds and growth factors in live food.

In summary, the results of this study indicate that striped bass larvae possess the ability to digest dietary lipids without a significant contribution of exogenous enzymes from *Artemia*. As of now, how feeding larvae hydrolyze the wax esters from the oil globule remains unknown. Future studies should concentrate on adaptational changes of lipolytic enzymes to compositional differences of lipids in the diet. Furthermore, phospholipid digestion and the importance of carboxyl ester lipase in fish appear to be areas of research that await closer attention.

CHAPTER 5

PREPARATION AND CHARACTERIZATION OF A COMPLEX MICROENCAPSULATED DIET FOR STRIPED BASS, *MORONE SAXATILIS* LARVAE

Introduction

Most of our knowledge concerning the nutritional requirements of fish comes from studies conducted on adult or juvenile species. The feeding physiology and nutritional requirements of marine fish larvae, however, are not well understood. This is mainly due to difficulties in the delivery of biochemically defined diets to the larvae when used as the only food item. Marine fish larvae are notorious for not accepting artificial diets as first food. Live food, on the other hand, varies enormously in biochemical composition (Watanabe et al. 1983). A high surface area to volume ratio effects the water stability of micron sized particles. Acceptability, digestibility and water stability appear to be the critical factors determining the suitability of artificial diets for the study of nutritional requirements in fish larvae. To date, no single artificial food has been shown to overcome the aforementioned problems.

Various microbound, microcoated and microencapsulated diets have been developed for the culture of marine fish larvae (see Jones et al. 1993; Watanabe and Kiron 1994 for review). Microbound diets have found widespread use in the culture of larval marine organisms (Kanazawa et al. 1982; Teshima et al. 1982a; Kanazawa et al. 1989). However, excessive losses of low molecular weight (LMW) nutrients to

leaching have been consistently reported for microbound diets (Goldblatt et al. 1979; Lopez-Alvarado et al. 1994). Binding crystalline amino acids to alginate, carrageenan or zein resulted in 80-90% leaching within minutes after immersion in water (Lopez-Alvarado et al. 1994).

Microencapsulation has emerged as an alternative vehicle of nutrient delivery that may potentially overcome the restraints associated with nutrient leaching and low water stability. Various microencapsulation techniques have been used in the culture of larval *Crassostrea virginica* (Chu et al. 1982, 1987), *Penaeus vannamei* (Villamar and Langdon 1993), *Penaeus japonicus* (Jones et al. 1987) and *Solea solea* (Appelbaum 1985) in laboratory scale trials. Lipid wall capsules (LWC) are capable of retaining water soluble compounds such as vitamins and minerals, however, cannot carry high nutrient loads to completely meet nutritional needs (Langdon and Siegfried 1984). Protein wall (PWC) and Ca-alginate microcapsules, on the other hand, can be used to encapsulate particulates and water soluble nutrients, but rapidly release low molecular weight compounds upon hydration (Langdon 1983; Levine et al 1983). Recently, Villamar and Langdon (1993) reported a method for small scale production of complex alginate microcapsules that are capable of efficiently retaining both macro and micronutrients. In our exhaustive trials, however,

acceptability of complex alginate microcapsules by striped bass larvae was extremely low (unpublished observations).

In this chapter, the methodology for preparation, size distribution, *in vitro* digestibility and nutrient release kinetics of a complex microcapsule prepared by incorporating the lipid-wall capsules along with other dietary material in a cross-linked protein microcapsule is described. This complex diet has the capability of releasing the low molecular weight attractants, such as free amino acids, to stimulate ingestion by larval fish, while retaining highly water soluble vitamins and minerals in lipid-wall capsules, thus, preserving the dietary content.

Materials and methods

Preparation of lipid-wall capsules

Lipid-wall capsules (LWC) were prepared according to Langdon and Siegfried (1984). The lipid wall material was prepared by dissolving 5 g ethyl cellulose and 5 g stearic acid in 25 ml of chloroform and mixing it with 90 g of purified menhaden oil (Zapata Heynie, Reedsville VA). Chloroform was evaporated overnight at 40 °C under nitrogen. One g of aqueous core solution (vitamin+mineral mixture) at 40 °C was added to 3 g of lipid wall material (40 °C) and homogenized for 1-2 min (Ultra-Turrax T25, Ikea Works,

Cincinnati OH) to form an aqueous-in-lipid emulsion. The emulsion was allowed to harden at 5 °C for 15 min. An emulsifier solution was prepared by dissolving 2.5 % (w/w) acacia (gum arabic) in 1 M $CaCl_2$. A secondary emulsion was formed by burst mixing the aqueous-in-lipid emulsion 4-5 times (5 ml each) in hot (60-70 °C) acacia solution. After each mixing, the suspension was poured into 250 ml of chilled (5 °C) deionized water. Hardened lipid-wall capsules were allowed to separate overnight in a separatory funnel at 5 °C. The capsule slurry was collected on a 5 µm mesh Nytex® filter and stored as a slurry in a refrigerator until used.

Preparation of complex protein-wall capsules

Complex protein wall capsules (CPWC) were prepared by incorporating LWC containing vitamins and minerals into cross-linked protein-wall capsules. The cross-linking procedure was modified from Jones et al. (1975) and Langdon (1989). Casein was used as the protein wall material due to its widespread use in feeding trials as a standard source of protein (Teshima et al. 1982b; Koshio et al. 1989). Four g of casein were dissolved in 100 ml of 0.02 M NaOH solution containing 0.2 % (w/v) urea. Four to five g of LWC and 10 g of dietary material (menhaden meal, starch and attractants) were added to the casein solution and mixed thoroughly. An organic solvent consisted of 1 % (v/v) adipoyl chloride and

2 % (w/v) crude soy lecithin in 250 ml of cyclohexane was prepared. The casein/diet mixture was atomized through a pressurized (20 psi N_2) nozzle unit (Wheaton Thin Layer Chromatography sprayer) into the swirling cyclohexane solution (Figure 1). The atomized particle size was controlled by two pressure valves. The capsule suspension was allowed to harden for 20 min. The microcapsules were allowed to settle in the beaker. The supernate was decanted and the capsules were poured into fresh cyclohexane. The remaining adipoyl chloride was removed by washing several times with fresh cyclohexane. The capsules were freeze dried and dry sieved through 300 µm and collected on 70 µm mesh nytex filters.

Size distribution of LWC and CPWC

Size distributions of LWC and CPWC were determined in a pre-calibrated multichannel particle counter (Coulter Counter TAII, Coulter Electronics, FL) at 280 and 560 µm aperture openings, respectively. Approximately 5-10 mg of LWC or CPWC were hydrated in 200 ml of Isotone®. Size distribution was measured at 10 second intervals and expressed as percentage of the total count.

Release of lysine from LWC, PWC and CPWC

Crystalline amino acid lysine was chosen to determine the leaching rate of LMW nutrients, because it is highly

water soluble and is essential for various marine and fresh water fishes (Wilson 1989). Release of lysine was determined in LWC, PWC and CPWC using ninhydrin reagent (Doi et al. 1981). Fifty µl of sample were placed in a microtiter plate well and added with 150 µl of ninhydrin reagent containing 0.8 g ninhydrin in 80 ml of ethanol (95%), 10 ml of glacial acetic acid and 1 g/ml $CdCl_2$. Purified lysine was used as a standard and applied to the plate along with the samples. The plate was incubated at 85 °C for 10 min and absorbance was read at 540 nm in a microtiter plate reader (Titertek, ICN Biomedicals, Costa Mesa, CA). Lipid-wall capsules containing lysine were prepared as above by dissolving 100 mg crystalline lysine in distilled water. The LWC were washed three times with 100 ml deionized water to remove the unencapsulated lysine. The CPWC were prepared by incorporating the LWC containing lysine into the cross-linked casein wall capsules. The conventional protein-wall capsules (PWC) were prepared by encapsulating 100 mg lysine (in 10 ml of casein solution) in the cross-linked protein wall membrane. The release rates of low molecular weight (LMW, amino acid lysine) compounds from conventional (PWC) and complex protein-wall capsules (CPWC) were compared.

Approximately 100 mg wet weight of LWC, containing 150 µg of lysine per mg wet weight, were added on a 1x25 cm

glass chromatography column containing 10 ml deionized water. The column was gently mixed for a few seconds to provide an even distribution of LWC. The LWC were allowed to float for 30 min and one ml of LWC free water was removed from the bottom of the column which had beed plugged with cotton wool. This was designated as t_0. A one ml aliquot was removed from the column at t_{24}h and 50 µl were used for determination of lysine. The difference between t_0 and t_{24} indicated the amount of lysine released from the LWC. The release rate of lysine was expressed as a percentage of the total lysine contained in the capsules added to the water column.

Release of lysine from PWC and CPWC were determined for 2 h after immersion in water. Five mg of PWC or CPWC containing 236 and 30 µg lysine, respectively, were placed in a microcentrifuge tube and 1 ml of deionized water was added. The capsule suspensions were hand shaken and centrifuged for 1 min at 1,000 rpm (Eppendorf microcentrifuge). Fifty µl of supernatant were removed at 0, 5, 15, 30, 60 and 120 min after hydration of the freeze dried CPWC and PWC for the detection of lysine. The capsule suspensions were kept on a shaker during the experiment. Total lysine contents of the capsules were determined after the disruption of the capsules in a homogenizer and a sonicator bath. The release of lysine was expressed as a

percentage of the total present in the capsules.

In vitro digestibility of complex protein-wall capsules

In vitro digestibility of PWC was determined using purified pepsin (porcine stomach mucosa), trypsin (porcine pancreas) (Sigma Chem. Co., St Louis, MO), or a crude enzyme extract prepared from 20 day old striped bass larvae. A batch of PWC containing no free amino acids, and casein as the only source of amino acids, were prepared to determine the breakdown of the cross-linked protein wall of the capsules. Five mg of freeze dried PWC free of amino acids, were suspended in 1 ml of 0.1 M acetate buffer, pH 5.0, and 0.1 M of Tris buffer, pH 8.0 for peptic and tryptic digestion, respectively. Various concentrations (0, 0.5, 1, 2 and 4 mg) of bovine pancreatic pepsin or trypsin were added to the suspension and incubated for one h at room temperature. Digestibility was determined as µg of amino acid produced (lysine equivalent) per hour using ninhydrin reagent. Blank incubations omitting the enzymes were run in parallel for all *in vitro* assays as controls.

Approximately 250 mg wet weight of striped bass, *Morone saxatilis,* larvae (20 days post-hatching) were homogenized in 0.1 M tris buffer, pH 7.0. The homogenate was centrifuged for 30 min at 20,000 g (Eppendorf microcentrifuge) and the supernate was used for the assays.

Various quantities (0, 0.2, 0.4, 0.8 and 1.6 mg protein) of enzyme extract were added to 5 mg/ml PWC suspension in the same buffer. The incubation was carried out for 20 h. Proteolytic digestion was determined as µg amino acid produced per hour (lysine equivalent) using ninhydrin reagent. The reaction was linear up to 24 h (R^2=0.97).

Digestibility of CPWC by 13 days post-hatching larvae was also observed microscopically using an Olympus BH2 microscope equipped with a SC-35 Olympus camera. Photomicrographs were taken at 50x.

Statistics

All assays were conducted in at least triplicate. Data were expressed as mean ± s.e. One way ANOVA was employed to determine the effects of microcapsule type on the release of lysine. Regression analyses were conducted using the SYSTAT (Systat, Inc., Evanston. IL) statistical package.

Results

Size distribution

The atomization unit designed for the gas-nozzle extrusion of microparticles (Fig.1) was be effective in the production of uniformly sized microcapsules. Over 80% of the protein-wall capsules were between 130 and 200 µm, with a mean diameter of 153 µm (Fig 2b). The process facilitated

the rapid encapsulation of the LWC in the cross-linked protein-wall capsules before the partitioning of the lipids to the cyclohexane phase. Fig. 3b shows a typical complex microcapsule with LWC encapsulated in a cross-linked hemoglobin membrane. Lipid-wall capsules had a wider range of size with a mean diameter of 35 μm. Fig 3a shows the LWC with singular or multiple aqueous core droplets embedded in a lipid/ethyl cellulose matrix. The encapsulation efficiency of lysine into LWC was calculated from the lysine content of the upper methanol/water phase after the extraction of the LWC paste with chloroform:methanol:water (2:2:1 by volume). Aqueous core of LWC formed 18.9% (± 1.6 w/w) of the total capsule.

Release of lysine from capsules

Release of lysine from conventional PWC and CPWC is shown in Fig 4. Nearly 45% of the total lysine leached out of PWC within 5 min after hydration. The loss of lysine steadily increased with time, reaching 70% and 99%, 30 min and 2 h after hydration, respectively. Lipid-wall capsules retained >90% of their lysine content after 24 h. The release of lysine from CPWC was significantly lower ($p<0.05$) than that observed with PWC. Approximately 20% of the total lysine was detected in the supernate after 15 min of suspension in water. The release rate remained relatively constant, not exceeding 30% after 2 h.

In vitro digestibility

Assays using crude enzyme extracts from 20 days post-hatching striped bass larvae or purified porcine trypsin and pepsin indicated that CPWC were susceptible to *in vitro* enzymatic digestion. Incubation of CPWC with increasing amounts of pepsin or trypsin resulted in increased accumulation of free amino acids in the digestion media (Fig 5). Both the tryptic and peptic digestion produced the same amount of amino acids. Preliminary studies indicated that the reactions were linear with time (R^2=97%, 98%). Crude enzyme extract was capable of breaking down the cross-linked protein wall (Fig 6). The rate of breakdown increased linearly (R^2=99%) with the increased amounts of enzyme protein in the suspensions.

Figure 1. Gas-nozzle extrusion unit used in the preparation of complex protein-wall capsules.

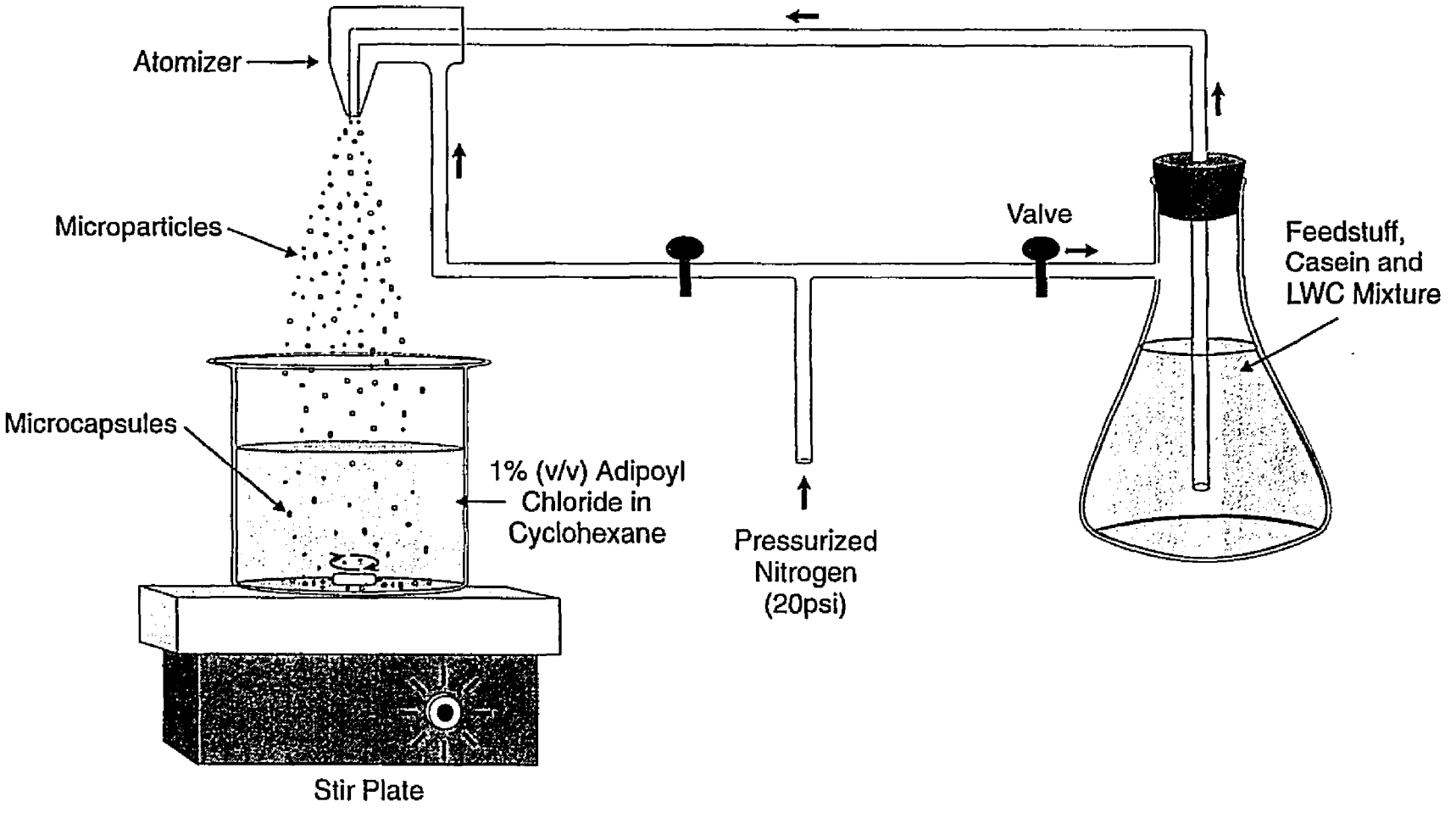

Atomizer
Microparticles
Microcapsules
1% (v/v) Adipoyl
Chloride in
Cyclohexane
Stir Plate
Pressurized
Nitrogen
(20psi)
Valve
Feedstuff,
Casein and
LWC Mixture

Figure 2. Percent size distribution of a) lipid-wall (LWC), and b) complex protein-wall capsules (CPWC). The number in x axis represent the mean diameter (μm) of each channel. Values are mean of triplicate counts.

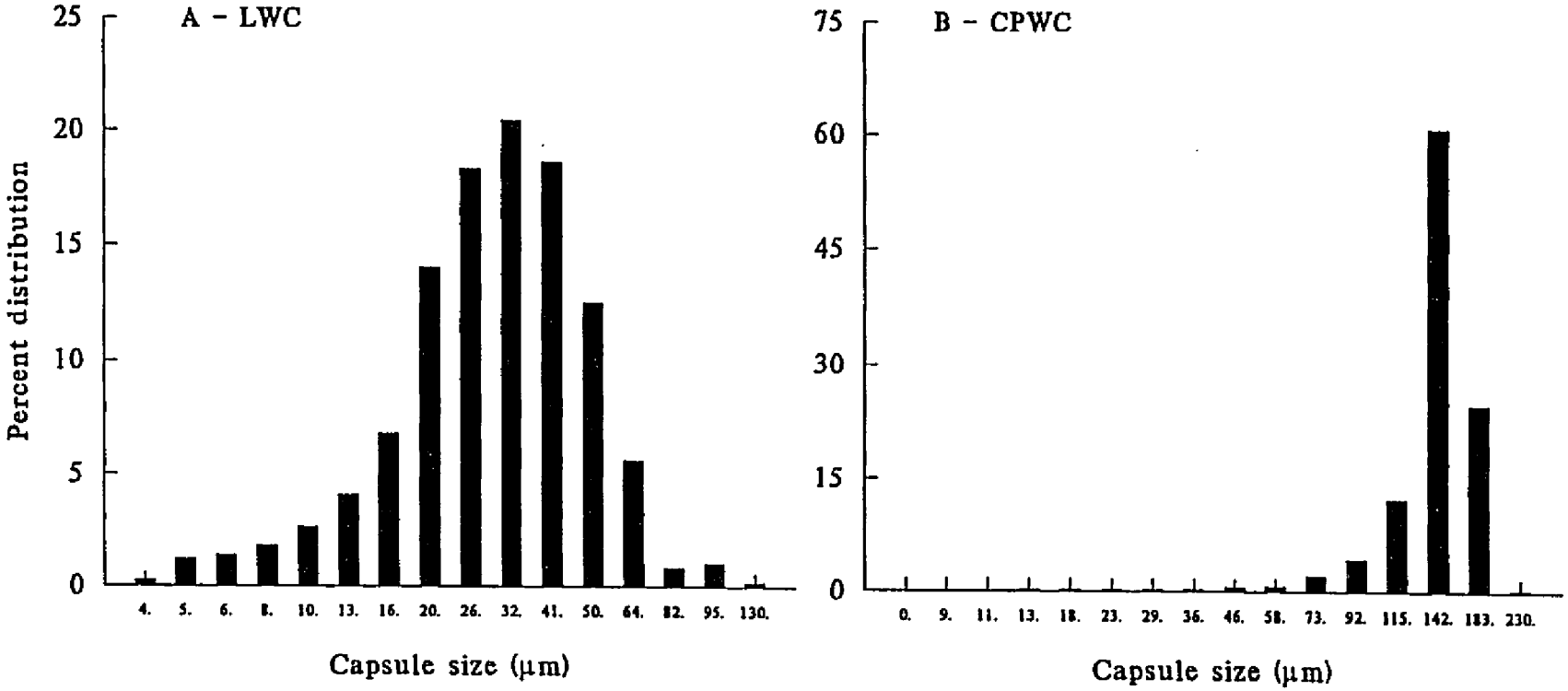
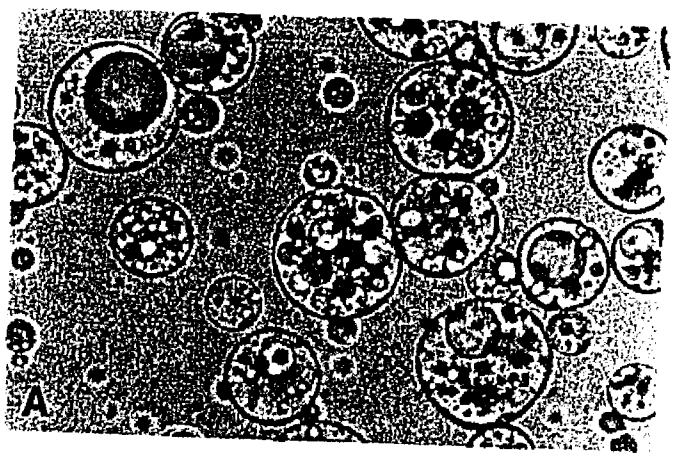

A - LWC
Percent distribution
25
20
15
10
5
0
4. 5. 6. 8. 10. 13. 16. 20. 26. 32. 41. 50. 64. 82. 95. 130.
Capsule size (µm)
B - CPWC
75
60
45
30
15
0
0. 9. 11. 13. 18. 23. 29. 36. 46. 58. 73. 92. 115. 142. 183. 230.
Capsule size (µm)

Figure 3. Photomicrographs of a) lipid-wall capsules (100x), b) complex protein-wall capsules (100x) and c) digestive tract of striped bass larva (40x).


A

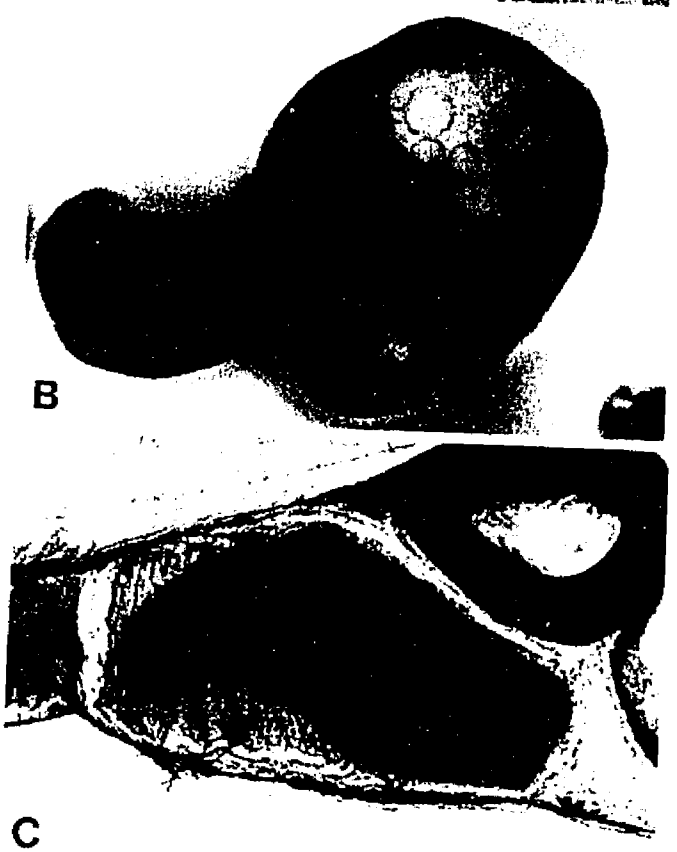

B
C

Figure 4. Percent release of lysine from conventional protein-wall capsules (PWC) and complex protein-wall capsules (CPWC) after rehydration in water. Values are means of triplicate measurements ± se.

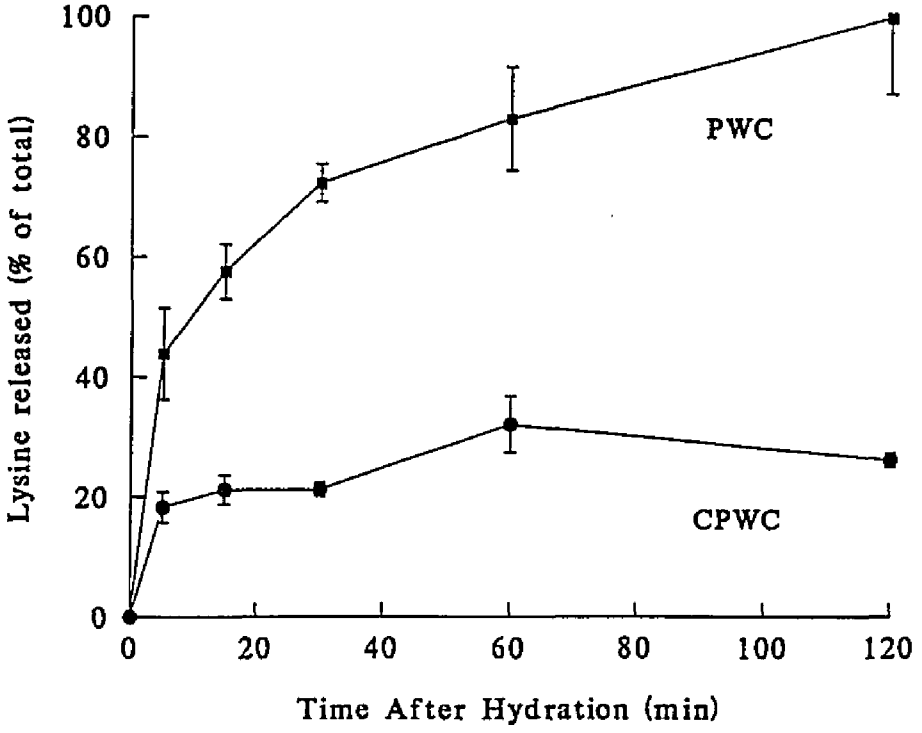

100
80
60
40
20
0
Lysine released (% of total)
PWC
CPWC
0
20
40
60
80
100
120
Time After Hydration (min)

Figure 5. *In vitro* digestibility of complex protein-wall capsules (µg amino acid produced/h, measured as lysine equivalent) by purified trypsin and pepsin. Values are means of triplicate measurements ± se.

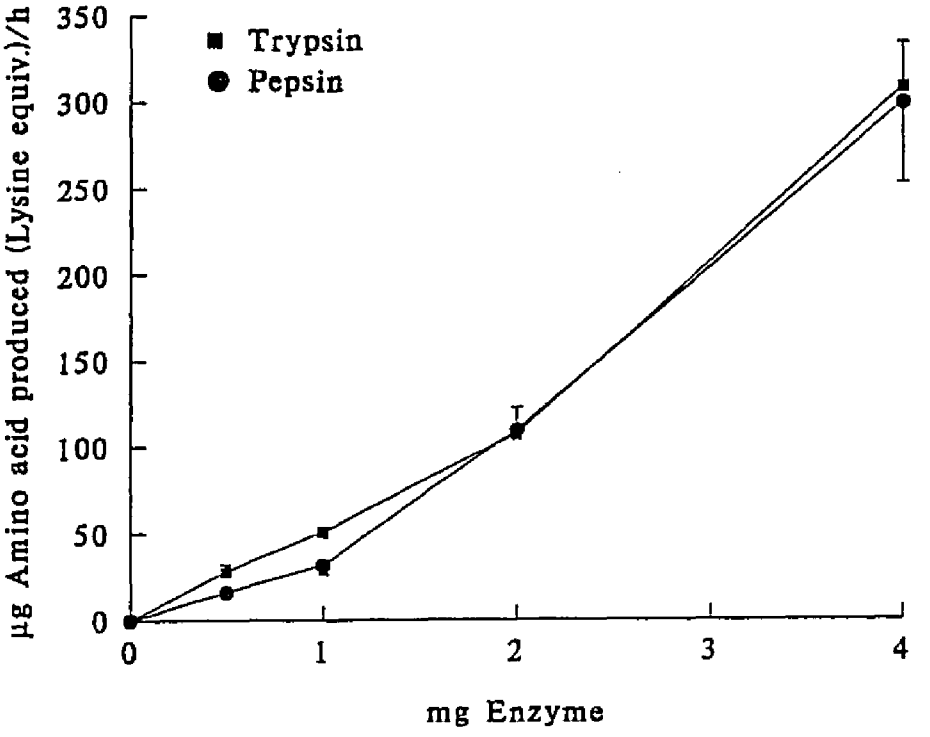

Trypsin
Pepsin
µg Amino acid produced (Lysine equiv.)/h
350
300
250
200
150
100
50
0
0
1
2
3
4
mg Enzyme

Figure 6. *In vitro* digestibility of complex protein-wall capsules by the enzymes extracted from 20 days post-hatching striped bass larvae (µg amino acid produced/h, measured as lysine equivalent). Values are means of triplicate measurements ± se.

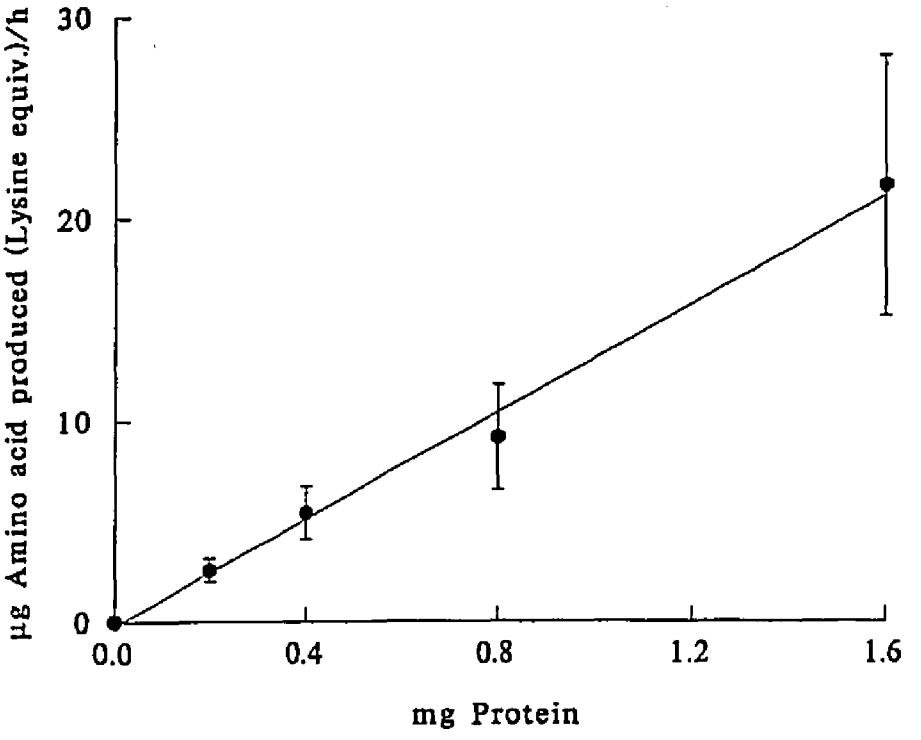
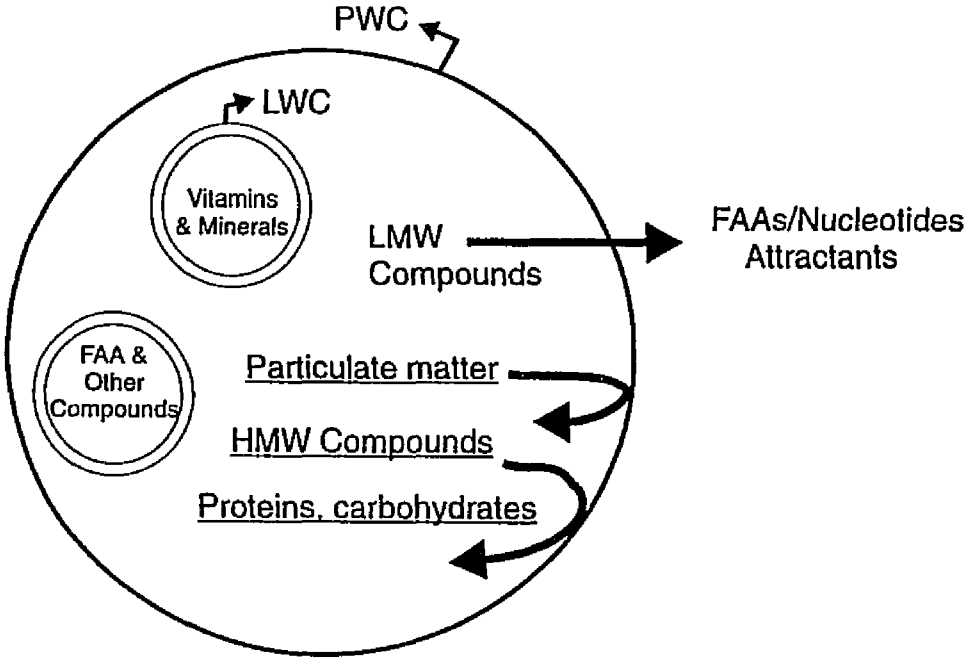

30
20
10
0
µg Amino acid produced (Lysine equiv.)/h
0.0
0.4
0.8
1.2
1.6
mg Protein

Figure 7. A model microencapsulated diet for marine fish larvae. Modified from Gabbott et al. 1976. PWC; protein-wall capsule, LWC; lipid-wall capsule, LMW; low molecular weight, HMW, high molecular weight, FAA; free amino acids.


PWC
LWC
Vitamins & Minerals
LMW Compounds
FAAs/Nucleotides Attractants
FAA & Other Compounds
Particulate matter
HMW Compounds
Proteins, carbohydrates

Discussion

The results of this study demonstrated that the complex microencapsulated diet can effectively retain low molecular weight, highly water soluble nutrients for 24h following immersion in water. In the present study, the encapsulation of lysine in CPWC significantly reduced the release of this compound compared to conventional PWC. The complex microcapsules, CPWC, released 25% of their lysine content after 2 h, while 100% release occurred with PWC. Villamar and Langdon (1993) reported that complex alginate microcapsules retained 84.7% of their ^{14}C-glucose activity after 18 h of suspension in seawater. High molecular weight compounds such as proteins are known to be efficiently retained (>95%) in protein-wall capsules when suspended in seawater for 24 h (Langdon 1989).

The type of protein used in the preparation of PWC has a profound effect on the properties of capsules (Levy and Andry 1991). In the present study, casein was used as a source of protein because of its widespread use in feeding studies as a reference protein (Teshima et al. 1982b; Koshio et al. 1989). Hemoglobin-wall complex microcapsules were found to be less acceptable by striped bass larvae (Chapter V) and were, therefore, excluded in the present study. Levy and Andry (1981) compared the release of salicilate from

various protein and mixed wall capsules and found that the wall composition significantly influenced the release rates. They attributed the differences in release rates to hydration time of the capsules; as the hydration time increased, the leaching rate dropped. The addition of small amounts of gelatin may aid in the formation of a denser inner core through gelation and may possibly reduce leaching. Moreover, slowly hydrating capsules are likely to remain suspended in the water column for longer periods of time, thus increasing the in availability to the larvae.

Sebacoyl chloride and 1,3,5-benzenetricarbonyl chloride have been used as interfacial cross-linking agents for the preparation of protein-wall capsules (Langdon 1989). In my preliminary trials, both agents resulted in very brittle wall formation and poor quality capsules. The best results were obtained with adipoyl chloride at 1% v/v in cyclohexane. Furthermore, the inclusion of small amounts of urea significantly reduced clumping. There was virtually no clumping of capsules during preparation, after freeze drying or rehydration. It should be noted that residual urea may have toxic effects on the larvae.

The current literature is quite deficient concerning *in vitro* and *in vivo* studies on digestibility of artificial diets by larval fishes. Walford et al. (1991) showed that

the fluorescent protein wall of all-protein-wall microcapsules was not broken down in the intestine of larval *Lates calcarifer*. In the present study, microscopical examination of the digestive tracts of striped bass larva revealed that the cross-linked protein wall was broken down. The release of hemoglobin was clearly visible in the anterior and posterior sections of the gut (Fig 3c). Hemoglobin-wall microcapsules were used for microscopical illustration, due to their better contrast with the transparent digestive tract of the larvae than the casein-wall capsules. Furthermore, the *in vitro* assays indicated that CPWC were susceptible to enzymatic breakdown by purified porcine trypsin and pepsin or the crude enzyme extract from striped bass larvae. Striped bass larva is known to possess appreciable amounts of trypsin (Baragi and Lovell 1986) and lipase (Chapter III). However, purified pepsin and trypsin were more effective in digesting the CPWC than the crude enzyme extract. Langdon and DeBevoise (1990) reported a similar observation that purified proteases had higher activities in digesting the protein-walled capsules than the extracellular digestive enzymes from the style of the oyster, *Crassostrea gigas*. However, this could be the result of lower total proteolytic activities of the crude larval extracts. Nevertheless, the assimilation of ^{14}C labeled PWC by juvenile oysters, *C. gigas* was 40.2%. Cross-linking of protein may increase the resistance of the

capsule wall to digestion by extracellular proteases.

Main difference in the desired properties between the capsules prepared for the filter/raptorial feeding larvae (Gabbott et al. 1976; Jones et al. 1976; Chu et al. 1982;1987; Langdon 1989) and those for visual/chemosensory feeding fish larvae is the permeability of the capsule wall to LMW compounds. The results of this study have lead me to hypothesize a new model microencapsulated diet for larval fishes that is a modified version of the model described by Gabbott et al. (1976) for marine bivalves (Fig 7). In this model, the outer shell of the microcapsule is permeable to LMW compounds. The semipermeable nature of the protein membrane is a crucial feature that allows the release of LMW attractants, such as, free amino acids and nucleotides that trigger ingestion by fish larvae. Highly water soluble vitamins and minerals, however, are encapsulated in the LWC which are not permeable to these essential nutrients, thus, preserving the nutritional quality of the diet. Additionally, the capsule type proposed in this model allows the encapsulation of digestive enzymes and other compounds in separate compartments. This is particularly important for extending the shelf life of the diet. In a separate study, I investigated the acceptability of CPWC by first feeding striped bass larvae and found that 75% of the larvae accepted the diet as first food (Chapter 5).

In conclusion, the CPWC described in this study may be used to investigate the effects of feeding stimulants, exogenous digestive enzymes and other bioactive compounds in larval fishes.

CHAPTER 6

GROWTH AND SURVIVAL OF STRIPED BASS (*MORONE SAXATILIS*) LARVAE FED COMPLEX MICROENCAPSULATED DIETS

Introduction

Culture of marine fish larvae relies heavily on the use of live food organisms, such as rotifers and *Artemia* nauplii. Although live food used in larval culture is superior to any artificial diet tested to date, they possess certain disadvantages when used extensively. Firstly, the nutritional quality of live food varies significantly from batch to batch, as a result of changes in biochemical composition (Watanabe et al. 1983). Secondly, the production of live food is costly and time consuming, which is a primary concern in commercial aquaculture (Jones et al. 1993; Jones et al. 1987; Ehrlich et al. 1989). Lastly, the ability to adjust the size and composition of live food to meet the specific requirements of the larvae is limited (Jones et al. 1987).

The acceptability of artificial diets by first feeding marine fish larvae varies enormously and appears to be a barrier impeding the development of a nutritionally complete diet. Promising but limited success has been reported for marine fish larvae fed microparticulate or microencapsulated diets as partial or full replacements for live food (Adron et al. 1973; Kanazawa et al. 1982; Applebaum 1985; Leibowitz et al. 1987; Kanazawa et al. 1989). Kanazawa et al. (1982) showed that the growth and survival of red sea bream (*Chrysophrys major*) and Ayu (*Plecoglossus altivelis*)

improved markedly when fed either zein microcoated or nylon-protein microcapsules in addition to live rotifers. In a separate study, Atlantic silverside, *Menidia beryllina*, larvae fed microencapsulated diets containing freeze-dried Artemia survived relatively well, however, growth was severely retarded (Leibowitz et al. 1987). Previous studies indicated that striped bass larvae did not accept artificial diets as first food and feeding the larvae solely on formula diets resulted in total mortality and poor growth (Webster and Lovell 1990; Tuncer et al. 1990). However, it is still unclear whether poor survival and growth of striped bass larvae is due to low diet acceptability or nutritional incompleteness of the diet.

Chapter 5 described the preparation and characterization of a complex cross-linked protein wall microcapsule prepared by incorporating lipid-wall capsules containing water soluble nutrients into a cross-linked protein membrane along with other dietary nutrients. The advantage of this diet was its ability to release low molecular weight attractants (i.e., free amino acids, nucleotides) from the protein wall while retaining other essential nutrients, such as water soluble vitamins, in the lipid-wall capsules incorporated in the diet. The objective of this study was to determine the diet acceptability, growth and survival of striped bass larvae fed the complex

protein wall capsules as partial or full replacement of *Artemia*, 7 through 21 days post-hatching (DPH).

Materials and Methods

Larval fish culture

Two separate experiments were conducted to determine the growth and survival of striped bass larvae fed microencapsulated diets in full (100%) or partial (60%) replacement of *Artemia* nauplii. Conditions for both experiments were similar with the exception that the diets used and the number of treatment replicates were different between the two experiments. Five day post-hatched (DPH) striped bass larvae were obtained from Brookneal State Hatchery, Brookneal, VA (Experiment 1) and Hudson River Utilities' Hatchery, Verplanck, NY (Experiment 2). Approximately 1,800 larvae were transferred to conical tanks (60 liter) in a recirculating system. Culture water (21 °C, 3 ppt) was passed through a 5 µm filter and crushed oyster shells to eliminate uneaten food items, fecal particles and ammonia. Ammonia levels were <200 µM in all tanks throughout the experiments. The culture tanks were continuously aerated. The feeding was initiated at 7 DPH and the experiments were terminated at 21 DPH.

Diet preparation

The method for the preparation of complex cross-linked

protein wall capsules was described previously (Ozkizilcik and Chu, 1995). Briefly, 20 g of the dietary mixture were added to 100 ml of 0.2% (w/v) urea in 0.02 N NaOH and stirred until the casein dissolved. Lipid wall capsules containing vitamin and mineral mixtures (Tables 3 and 4) were prepared as described elsewhere (Langdon and Siegfried 1984; Chu et al. 1987) and added to the diet mixture (Table 1). The mixture was atomized into a swirling 1% (v/v) adipoyl chloride in cyclohexane solution containing 2% (w/v) crude lecithin. The microparticles were allowed to cross-link at the water-cyclohexane interface for 15 min. The slurry was poured into cyclohexane to quench the reaction. The capsules were washed with cyclohexane to remove excess adipoyl chloride and freeze dried for storage. This process produced cross-linked protein wall microcapsules at a mean diameter of 153 µm. The complex diet consisted of lipid wall capsules containing essential vitamins and minerals, and other dietary compounds (i.e. menhaden meal, starch) encapsulated within a cross-linked protein wall. An amino acid mixture resembling that of *Artemia* was included in the diets at 5% by weight (Table 2). The capsules consisted of 53% protein and 21% lipids as determined by analyses.

Feeding experiments

In Experiment 1, the treatments consisted of hemoglobin wall capsules (HWC), casein wall capsules (CWC),

HWC+*Artemia* (2 nauplii/ml), CWC+*Artemia* (2 nauplii/ml), 40% ration *Artemia* (2 nauplii/ml) and full ration *Artemia* (5 nauplii/ml). There was no replication within each treatment due to the limited availability of culture tanks. Microencapsulated diets were fed to the larvae at 2g/tank/day in four separate rations (0.5 g/ration) based on the dry weight of *Artemia* (Leger et al. 1986). The number of *Artemia* nauplii in the culture tanks that received full or 40% live food were checked regularly and necessary amounts were added.

In Experiment 2, each treatment was replicated (N=2) to determine whether there was any tank effect. The hemoglobin wall capsules were excluded from the second experiment due to high mortalities observed with these capsules in Experiment 1 (100% mortality at 17 DPH). In attempt to increase ingestion rate, casein wall capsules used in Experiment 2 were modified to contain water and lipid soluble extracts (modified CWC) of newly hatched *Artemia* nauplii. To prepare the extract, 30 g wet weight of *Artemia* were homogenized in 80% (v/v) ethanol. The homogenate was centrifuged and the upper phase was removed and dried in a rotary evaporator. Ethanol soluble residue was redissolved in distilled water and added to the dietary mixture. The residue was dried and lipids were extracted with chloroform:methanol (2:1 v/v). *Artemia* lipids were added to

the lipid mixture used in the preparation of lipid wall capsules. Treatments were similar to Experiment 1 and consisted of modified CWC, modified CWC+*Artemia* (2 nauplii/ml).

In both experiments, 40% ration *Artemia* (2 nauplii/ml), full ration *Artemia* (5 nauplii/ml) and unfed larvae served as the controls. Our previous trials showed that the growth of larvae was determined by the number of *Artemia* nauplii present in the culture water (unpublished observations). Therefore, 100% and 40% ration was determined to be 5 and 2 nauplii/ml.

The *Artemia* cysts hatched daily in 60 L transparent tanks in 1 µm filtered estuarine water (24 ppt) at 28 °C. Freshly hatched nauplii were collected on 150 µm filters, rinsed well and stored live at 10 °C for feeding.

Ingestion and breakdown of microencapsules by the larvae

Diet acceptability was measured microscopically on 7 and 14 DPH larvae in both experiments. Thirty min after feeding, 25 larvae were sampled and examined for the presence of food items in the digestive tract. Ingestion rate (%) was calculated as;

(# of larvae with food items in the gut / total # of larvae examined) x 100.

To determine the breakdown of complex microencapsulated diets, in Experiment 2, five larvae (13 DPH) were taken from the culture tanks approximately 30 min after feeding. Individual larvae (N=5) were placed in a multiwell tissue culture plate containing 2 ml of culture water. Each larva was transferred to a microscope slide with a Pasteur pipet at 0 min, 30 min, 1.5 and 3.5 hours and examined under a microscope. Photomicrographs were taken at 5x (Olympus BH2 microscope equipped with a SC-35 Olympus camera) to reveal the state of gut contents through the transparent body of the larva. No mortality was observed during the examination.

Larval fish measurement

Larvae were sampled from each tank at 20 DPH to determine growth and survival (Experiment 1; N=40/treatment, Experiment 2; N=25/replicate, N=50/treatment). Wet weights were determined to the nearest 0.1 mg on individual larva after blotting dry on a paper towel. Survival rates, expressed as percentages, were determined by counting the number of fish that survived at the end of the experiment.

Statistical analyses

Wet weight data were analyzed by ANOVA. In Experiment 2, no tank effect was noted (treatment replicate), therefore, the untransformed data were pooled and one-way ANOVA was employed. Pairwise comparisons were made with Tukey's test using the SYSTAT® statistical package.

Results

The results showed that the complex protein-wall microcapsules were readily ingested by the first feeding striped bass larvae (Table 5). In Experiment 1, the ingestion rates of live and microencapsulated diets ranged from 40 to 71%. Hemoglobin wall microcapsules had the lowest ingestion rate (40%), while full ration *Artemia* was consumed at the highest rate (71%). The ingestion rate of the casein wall microcapsules was slightly higher than that of the hemoglobin wall capsules. On 14 DPH, the ingestion rate of all diets increased markedly, reaching 85% in HWC and 100% in other treatments. In Experiment 2, the inclusion of *Artemia* extracts in the capsules significantly improved the ingestion rate of CWC. On 7 DPH, 75% of the larvae accepted CWC as first food. In the second experiment, the larvae seemed to ingest diets more readily than in the first experiment.

In Experiment 1 supplementing *Artemia* with both HWC and

CWC significantly increased ($p<0.05$) the wet weight of the larvae compared to unsupplemented *Artemia* (Table 6). The larvae fed *Artemia* (2 nauplii/ml)+CWC grew as fast as those fed full ration *Artemia* (5 nauplii/ml). Although the final wet weight (ww) of the larvae fed HWC supplemented diet was higher ($p<0.05$) than that of unsupplemented, the final weight gain was significantly less ($p<0.05$) than the full ration or CWC supplemented diet (Table 6). The larvae fed solely on HWC or CWC did not show any apparent growth. CWC fed larvae survived at a reduced rate (8.7%), while total mortality was recorded for HWC at 17 DPH. All unfed (starved) larvae were dead on 14 DPH. Survival rates for *Artemia* fed larvae ranged from 63 to 78%, full ration *Artemia* being the highest.

In Experiment 2, a similar but less dramatic growth was observed when *Artemia* (2 nauplii/ml) was supplemented with CWC containing water and lipid soluble fractions of *Artemia*, although the final wet weight was significantly higher than those fed 40% ration *Artemia* (2 nauplii/ml) ($p<0.01$). Larvae fed full ration *Artemia*, on the other hand, had significantly higher final ww than all other groups ($p<0.01$). When CWC was fed to the larvae as the only diet, there was no gain in ww at the end of the experiment. However, the survival rate at termination was significantly improved to 49%. Overall survival of *Artemia* fed diets was

very high.

Microscopic examination of the digestive tract of larvae at 30 min, 1, 2 and 4 h after feeding is illustrated in Fig 1. The digestive tract was full of capsules at 30 min. Nearly half of the ingested capsules dissipated after 1 h. The capsule aggregate shrank in size in the following three hours, almost completely residing in the anterior section of the gut. Small fragments of the digested capsules were observed in the posterior section of the digestive tract.

Table 1. Composition (% by weight) of cross-linked complex protein-walled microcapsules prepared as described in Material and Methods section.

	Cross-linked Protein-Walled Capsules	
	CWC (%)	HWC (%)
Casein	20	-
Hemoglobin	-	30
Menhaden meal	35	30
Starch	5	5
Amino acid mix[1]	5	5
Cholesterol	1	1
Lecithin	1	1
Lipid-walled capsules[2]	25	20
Attractants[3]	8	8

[1]The composition of amino acid mixture is given in Table 2.

[2]Lipid-walled capsules were prepared as described in Materials and Methods. Aqueous core of lipid-walled capsules contained 5g vitamin mix and 5g mineral mix dissolved in 20 ml of distilled water. The compositions of vitamin and mineral mixes are given in Table 3 and 4.

[3]Attractants included glycine (2g), betaine (2g), inosine (1g), inosine 5'-monophosphate (1g), guanosine (1g) and guanosine 5'-monophosphate (1g).

Table 2. Composition of amino acid mixture (from Seidel et al 1980 and Dabrowski and Rusiecki 1983)

Amino acid	%
Phenyl alanine	4.0
Tyrosine	4.4
Leucine	4.3
Isoleucine	2.9
Methionine	3.2
Valine	2.6
Alanine	8.0
Glycine	10.8
Glutamic acid	3.0
Proline	13.1
Serine	5.2
Threonine	4.7
Aspartic acid	3.0
Arginine	3.9
Histidine	3.5
Lysine	1.8
Cysteine	6.5
Tryptophan	4.5
Betaine	10.8
TOTAL	100

Table 3. Composition of vitamin mixture (from Teshima et al. 1982)

Vitamin	mg
p-Aminobenzoic acid	22.6
Biotin	0.3
Inositol	226.7
Nicotinic acid	45.3
Ca-pantothenate	15.9
Pyridoxine HCl	2.7
Riboflavin	11.3
Thiamine HCl	3.4
Menadione*	2.7
B-Carotene*	5.7
A-Tocopherol*	22.7
Cyanocobalamine	0.005
Calciferol*	0.6
Na-Ascorbate	113.3
Folic acid	0.9
Choline chloride	463.3

* fat soluble vitamins were dissolved in menhaden oil before the lipid-walled capsules were prepared. Quantities were adjusted according to the lipid composition of the diet.

Table 4. Composition of mineral mixture (from Teshima et al. 1982)

Main mineral	g	Trace mineral	mg
NaCl	0.435	$AlCl_3.6H_2O$	1.5
$MgSO_4.7H_2O$	1.37	$ZnSO_4.7H_2O$	30.0
$NaH_2PO_4.2H_2O$	0.872	CuCl	1.0
$KH_2PO_4.2H_2O$	2.398	$MnSO_4.4H_2O$	8.0
$Ca(H_2PO_4)_2.2H_2O$	1.358	KI	1.5
Ferric citrate	0.297	$CoCl_2$	10.0
Calcium lactate	3.27		

Table 5. Acceptability (%) of live and microencapsulated diets by striped bass larvae at first feeding (7 DPH) and 14 DPH.

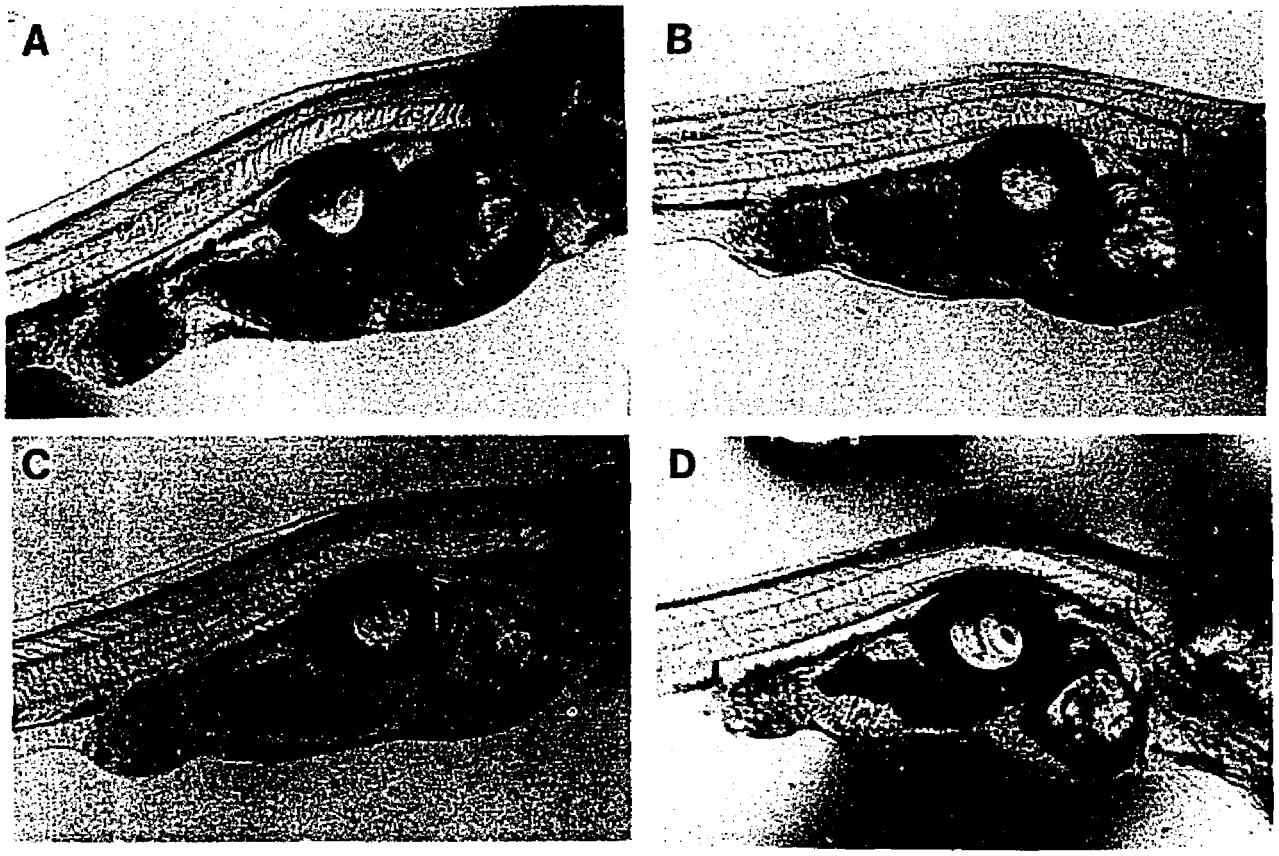
Treatments	Experiment 1		Experiment 2	
	7 DPH	14 DPH	7 DPH	14 DPH
Artemia (5 nauplii/ml)	71	100	98	100
Artemia (2 nauplii/ml)	60	100	96	100
CWC + Artemia (2 nauplii/ml)	65	100	96	100
CWC	46	100	75	100
HWC + Artemia (2 nauplii/ml)	70	100	-	-
HWC	40	85	-	-

Table 6. Growth and survival of striped bass larvae fed live and microencapsulated diets at 21 DPH. Initial indicates 7 DPH.

	Experiment 1		Experiment 2	
	Wet Weight (mg)	Survival (%)	Wet Weight (mg)	Survival (%)
Initial	$1.3^{a} \pm 0.0$	100	$0.9^{d} \pm 0.0$	100
Artemia (5/ml)	$8.8^{c} \pm 0.3$	78	$11.8^{a} \pm 0.4$	81
Artemia (2/ml)	$6.3^{d} \pm 0.2$	70	$7.6^{b} \pm 0.4$	88
CWC + Artemia (2/ml)	$8.9^{c} \pm 0.3$	63	$8.9^{c} \pm 0.2$	81
CWC	$1.3^{a} \pm 0.1$	9	$0.9^{d} \pm 0.0$	49
HWC + Artemia (2/ml)	$7.6^{b} \pm 0.2$	70		
HWC	*	0		

* All HWC fed larvae were dead on 17 DPH

Figure 1. Microscopical observation of microencapsulated diets in the digestive system of 13 days old striped bass larvae. A;30, B;60, C;120 and D:240 min after feeding.


A
B
C
D

Discussion

Protein wall microcapsules have been extensively used in the culture of marine organisms since their modification for aquatic filter feeders (Jones et al. 1974). Numerous studies have investigated the acceptability and digestibility of protein wall microcapsules by adult (Langdon 1989) and larval oysters (Chu et al. 1987), shrimp larvae (Jones et al. 1987) and fish larvae (Applebaum 1985; Kanazawa et al. 1982). Nevertheless, the use of microencapsulated diets has expanded to a commercial scale only in penaeid shrimp culture (Jones et al. 1987).

In the present study, we demonstrated that the partial replacement of *Artemia* with complex protein-wall microcapsules did not result in any significant deleterious effect, particularly in Experiment 1, on the survival and growth of first feeding striped bass larvae and subsequent development. Earlier studies reported that striped bass larvae did not accept artificial diets as first food (Webster and Lovell 1990; Tuncer et al. 1990). In the present study, the ingestion rate of complex protein wall capsules by first feeding larvae was promising (75% in Experiment 2). Once ingested, the capsules formed a large aggregate in the anterior section of the digestive tract (Fig 1). Microscopic examination of the larvae revealed that nearly half of the capsule aggregate dissipated within

1 h of feeding. The remaining aggregate shrank in size in the following three hours, suggesting slow digestion. Langdon (1989) reported a 40% ^{14}C absorption efficiency of cross-linked protein wall capsules by *Crassostrea gigas*, which was lower than reported for live food. In the present study, when fed solely, complex protein wall capsules did not support any growth of the larvae. However, a high survival rate (49%) was obtained in the second experiment. Similar results were reported in *Menidia beryllina* (Leibowitz et al. 1987), *P. altivelis* and *P. major* (Kanazawa et al. 1982) that microencapsulated diets did not support any growth, however, the larvae survived relatively well.

It has been consistently reported that the digestibility of microencapsulated diets increased when live food was offered as a supplement (Walford et al. 1991; Kanazawa et al. 1982). The poor growth of fish larvae fed artificial diets has been attributed to the lack of digestive enzymes in the larvae (Dabrowski 1979). It has been hypothesized that the initial digestive processes in the rudimentary digestive tract of the fish larvae are facilitated by the enzymes present in live food (Dabrowski 1979). Although striped bass larvae possess tryptic (Baragi and Lovell 1986) and lipolytic enzymes (Ozkizilcik et al. 1995) at first feeding, dietary enzymes may assist in digestion of artificial diets or stimulate enzyme secretion

from fish larvae. In this context, complex microencapsulated diets offer a potential method for the application of dietary enzymes in larval diets. Proteases and amylases can be ecapsulated in LWC as separate compartments to increase the shelf life of the diet. Maugle et al. (1983) reported that dietary supplements of microencapsulated alpha-amylase and bovine trypsin at levels comparable to those of live short-necked clam enhanced the growth of shrimp *Penaeus japonicus*. It should be noted that the digestibility of different proteins varies considerably among species. The nutritional value of casein for larval *Penaeus japonicus* was studied by Koshio et al. (1989). When crab protein was used as the only protein source, growth and survival of *P. japonicus* larvae were significantly reduced. Addition of casein in the diets markedly improved the growth and survival. Although, our *in vitro* studies showed that the protein wall of capsules were digestible by the enzymes extracted from the larvae (Chapter 4), the microencapsulated diet may not be utilized as efficiently as live food, perhaps due to the apparent differences in amino acid composition between *Artemia* and casein (Seidel et al. 1980; Teshima et al. 1986). Hemoglobin has been suggested to be a poor source of protein due to its resistance to proteolytic digestion (D.A. Jones, personal communication). Beside the low digestibility of the cross-linked protein wall, the involvement of toxic organic solvents, such as cyclohexane,

in diet preparation may reduce the nutritional value of protein-walled capsules to striped bass larvae compared to live food. Although it is believed that freeze-drying efficiently removes toxic solvents (Langdon 1989), an in vitro cytotoxic activity of cross-linked protein microcapsules on the human erythroleukaemic cells has been reported (Desoize et al. 1989). The capsules inhibited the growth of the cells in a concentration dependent manner. However, the cytotoxic effect was non-specific, reversible and dependent on the contact with the cell membrane.

Further improvement of the microencapsulated diet used in the present study may overcome the three major obstacles, associated with artificial food, namely, low acceptability, low digestibility and nutritional incompleteness. The semipermeable nature of the cross-linked protein-wall allows the release of low molecular weight attractants, such as free amino acids to stimulate ingestion by larvae, while the highly water soluble nutrients are retained in the lipid wall capsules. Previous studies reported that in the presence of live food and artificial diets *Sparus aurata* larvae preferentially consumed live food (Fernandez-Diaz et al. 1994). In our study, microscopical examination of the larvae revealed that striped bass larvae consumed both the microencapsulated diets containing attractants and the *Artemia* nauplii when both were present in the water column.

Inclusion of lipid and water soluble extracts of *Artemia* further improved the ingestion rate of capsules by fish larvae, suggesting that the phagostimulants used in the diet preparation were effective. This was in accordance with our preliminary results that suggested that striped bass larvae feed primarily by chemical stimulus, rather than visual (unpublished observations). When the larvae were placed in a container in complete darkness, 97 % of the larvae were able to capture *Artemia* nauplii.

In conclusion, this study revealed that complex protein-wall capsules are readily ingested by striped bass larvae and can be used as partial supplement for live food in the culture of larvae. Additionally, the complex protein-wall capsules can potentially be used as a vehicle of nutrient delivery to study the nutritional requirements and feeding physiology of marine fish larvae. Furthermore, the nutrient assimilation efficiency from capsules is not known and needs to be studied using radiolabeled compounds. Future studies should focus on the development of non-cross-linked protein wall capsules and improving the digestibility of microencapsulated diets through the application of digestive enzymes.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The results of the studies described in this dissertation have led to the development of two new bioencapsulation and microencapsulation techniques that could potentially open new areas of research in larval nutrition. Furthermore, clarifications have been made for the lipolytic capacity of the digestive system of striped bass larvae, the contribution of the dietary enzymes to the total lipolytic processes, and the dietary PUFA requirements during early development.

The following summarizes the major findings outlined in the previous chapters of this dissertation.

A. The results detailed in Chapter II revealed that the three enrichment techniques were equally effective in enriching *Artemia* with PUFA. Eicosapentaenoic acid content of the nauplii appeared to be the critical factor in determining the growth of striped bass larvae. There was an indication of limited bioconversion of shorter-chain length PUFA to long-chain PUFA. Therefore, it is advisable that the fatty acid composition of *Artemia* be determined prior to feeding the larvae and appropriate enrichment be employed when necessary. Among all the enrichment techniques investigated in this study, special emphasis should be placed on the microencapsulated diets, because of their ease of application, reasonable costs, minimal contamination of

the enrichment media by bacteria and their ability to encapsulate a wide variety of nutrients.

B. The results of chapter III suggest that liposomes emerge as a new technique to enrich live diets with phospholipids and free amino acids, and possibly bioencapsulate therapeutic drugs for marine fish larvae. Future studies on this subject should investigate the use of marine origin phospholipids, rich in n-3 PUFA to enrich *Artemia* with these fatty acids along with other nutrients, such as essential amino acids and bioactive compounds.

C. The long advocated hypothesis that initial digestion in fish larvae proceeds by the action of enzymes present in live food (Dabrowski, 1979) is challenged in Chapter IV. The results indicated that the contribution of dietary enzymes to the total lipolytic processes was minimal (<6.5%) and the first feeding larvae had the capacity to digest 47% of their daily lipid consumption. Although this estimate appears to be low, it should be noted that the enzyme measurements reflect the resting state when the digestive tract was empty. The lipolytic activities are likely to be higher when the digestive system is stimulated by the diet. In conclusion, future studies should concentrate on adaptational changes of the lipolytic enzymes to compositional differences of lipids in diet. Additionally,

phospholipid digestion in fish appears to be unknown and awaits further investigation.

D. The method for small-scale production of a complex microencapsulated diet, detailed in Chapter V, is one of the major accomplishments of this dissertation. The complex protein-walled capsules were stable in water, readily acceptable, digestible and had extended shelf-life. The microencapsulated diet developed in this study offers a great potential for the; a) study of the species-specific phagostimulants in fish larvae; b) assessment of the dietary requirements and c) application of digestive enzymes. Nevertheless, further improvements are needed in retention characteristics through the use of liposomes and the development of non cross-linked protein-wall capsules that are readily digestible by the larvae.

E. The study described in Chapter VI is the first attempt to raise striped bass larvae on microencapsulated diets as partial replacement of live food *Artemia*. The microencapsulated diet used in this study was readily accepted by striped bass larvae and promoted growth of the larvae without any marked deleterious effect when supplemented with *Artemia*. However, feeding the larvae solely on microencapsulated diets resulted in reduced survival and poor growth. Further studies are needed to

determine the reasons underlying the nutritional incompleteness of these diets. Application of growth factors, such as pituitary hormones, may be essential to stimulate growth comparable to that observed with live food.

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